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<p>(54) Title: NOVEL METHODS OF IMAGING AND TREATMENT WITH TARGETED COMPOSITIONS</p> <p>(57) Abstract</p> <p>Novel ultrasound methods comprising administering to a patient a targeted vesicle composition which comprises vesicles comprising a lipid, protein or polymer, encapsulating a gas, in combination with a targeting ligand, and scanning the patient using ultrasound. The scanning may comprise exposing the patient to a first type of ultrasound energy and then interrogating the patient using a second type of ultrasound energy. The targeting ligand preferably targets tissues, cells or receptors, including myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein GPIIbIIIa receptor. The methods may be used to detect a thrombus, enhancement of an old or echogenic thrombus low concentrations of vesicles and vesicles targeted to tissues, cells or receptors.</p>			

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**NOVEL METHODS OF IMAGING AND
TREATMENT WITH TARGETED COMPOSITIONS**

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Field of the Invention

The present invention relates to novel methods of imaging involving targeted compositions. More particularly, the present invention relates to novel targeted compositions which may be targeted to tissues in the body for diagnostic imaging and/or for therapeutic applications.

Background of the Invention

A variety of imaging techniques have been used to diagnose diseases. Included among these imaging techniques is X-ray imaging. In X-rays, the images produced reflect the different densities of structures and tissue in the body of the patient.

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- To improve the diagnostic usefulness of this imaging technique, contrast agents may be employed to increase the density of tissues of interest relative to surrounding tissues. Examples of such contrast agents include, for example, barium and iodinated compounds, which may be used for X-ray studies of the gastrointestinal region, including the
- 5 esophagus, stomach, intestines and rectum. Contrast agents may also be used for computed tomography (CT) and computer assisted tomography (CAT) studies to improve visualization of tissue of interest, for example, the gastrointestinal tract.

Magnetic resonance imaging (MRI) is another imaging technique which, unlike X-rays, does not involve ionizing radiation. MRI may be used for producing cross-
10 sectional images of the body in a variety of scanning planes such as, for example, axial, coronal, sagittal or orthogonal. MRI employs a magnetic field, radio frequency energy and magnetic field gradients to make images of the body. The contrast or signal intensity differences between tissues mainly reflect the T1 (longitudinal) and T2 (transverse) relaxation values and the proton density, which generally corresponds to the free water
15 content, of the tissues. To change the signal intensity in a region of a patient by the use of a contrast medium, several possible approaches are available. For example, a contrast medium may be designed to change either the T1, the T2 or the proton density.

Generally speaking, MRI requires the use of contrast agents. If MRI is performed without employing a contrast agent, differentiation of the tissue of interest from
20 the surrounding tissues in the resulting image may be difficult. In the past, attention has focused primarily on paramagnetic contrast agents for MRI. Paramagnetic contrast agents involve materials which contain unpaired electrons. The unpaired electrons act as small magnets within the main magnetic field to increase the rate of longitudinal (T1) and transverse (T2) relaxation. Paramagnetic contrast agents typically comprise metal ions, for
25 example, transition metal ions, which provide a source of unpaired electrons. However, these metal ions are also generally highly toxic. In an effort to decrease toxicity, the metal ions are typically chelated with ligands.

Metal oxides, most notably iron oxides, have also been employed as MRI contrast agents. While small particles of iron oxide, for example, particles having a
30 diameter of less than about 20 nm, may have desirable paramagnetic relaxation properties, their predominant effect is through bulk susceptibility. Nitroxides are another class of

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MRI contrast agent which are also paramagnetic. These have relatively low relaxivity and are generally less effective than paramagnetic ions.

The existing MRI contrast agents suffer from a number of limitations. For example, increased image noise may be associated with certain contrast agents, including

5 contrast agents involving chelated metals. This noise generally arises out of intrinsic peristaltic motions and motions from respiration or cardiovascular action. In addition, the signal intensity for contrast agents generally depends upon the concentration of the agent as well as the pulse sequence employed. Absorption of contrast agents can complicate interpretation of the images, particularly in the distal portion of the small intestine, unless

10 sufficiently high concentrations of the paramagnetic species are used. *See, e.g., Kornmesser et al., Magnetic Resonance Imaging, 6:124 (1988).*

Other contrast agents may be less sensitive to variations in pulse sequence and may provide more consistent contrast. However, high concentrations of particulates, such as ferrites, can cause magnetic susceptibility artifacts which are particularly evident,

15 for example, in the colon where the absorption of intestinal fluid occurs and the superparamagnetic material may be concentrated.

Toxicity is another problem which is generally associated with currently available contrast agents, including contrast agents for MRI. For example, ferrites often cause symptoms of nausea after oral administration, as well as flatulence and a transient

20 rise in serum iron. The gadolinium ion, which is complexed in Gd-DTPA, is highly toxic in free form. The various environments of the gastrointestinal tract, including increased acidity (lower pH) in the stomach and increased alkalinity (higher pH) in the intestines, may increase the likelihood of decoupling and separation of the free ion from the complex.

Ultrasound is another valuable diagnostic imaging technique for studying

25 various areas of the body, including, for example, the vasculature, such as tissue microvasculature. Ultrasound provides certain advantages over other diagnostic techniques. For example, diagnostic techniques involving nuclear medicine and X-rays generally involves exposure of the patient to ionizing electron radiation. Such radiation can cause damage to subcellular material, including deoxyribonucleic acid (DNA),

30 ribonucleic acid (RNA) and proteins. Ultrasound does not involve such potentially damaging radiation. In addition, ultrasound is relatively inexpensive relative to other

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diagnostic techniques, including CT and MRI, which require elaborate and expensive equipment.

Ultrasound involves the exposure of a patient to sound waves. Generally, the sound waves dissipate due to absorption by body tissue, penetrate through the tissue or reflect off of the tissue. The reflection of sound waves off of tissue, generally referred to as backscatter or reflectivity, forms the basis for developing an ultrasound image. In this connection, sound waves reflect differentially from different body tissues. This differential reflection is due to various factors, including the constituents and the density of the particular tissue being observed. Ultrasound involves the detection of the differentially reflected waves, generally with a transducer that can detect sound waves having a frequency of one megahertz (MHZ) to ten MHZ. The detected waves can be integrated into an image which is quantitated and the quantitated waves converted into an image of the tissue being studied.

As with the diagnostic techniques discussed above, ultrasound also generally involves the use of contrast agents. Exemplary contrast agents include, for example, suspensions of solid particles, emulsified liquid droplets, and gas-filled bubbles. See, e.g., Hilmann et al., U.S. Patent No. 4,466,442, and published International Patent Applications WO 92/17212 and WO 92/21382. Widder et al., published application EP-A-0 324 938, discloses stabilized microbubble-type ultrasonic imaging agents produced from heat-denaturable biocompatible protein, for example, albumin, hemoglobin, and collagen.

The quality of images produced from ultrasound has improved significantly. Nevertheless, further improvement is needed, particularly with respect to images involving vasculature in tissues that are perfused with a vascular blood supply. Accordingly, there is a need for improved ultrasound techniques, including improved contrast agents which are capable of providing medically useful images of the vasculature and vascular-related organs.

The reflection of sound from a liquid-gas interface is extremely efficient. Accordingly, bubbles, including gas-filled bubbles, are useful as contrast agents. The term "bubbles", as used herein, refers to vesicles which are generally characterized by the presence of one or more membranes or walls surrounding an internal void that is filled

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with a gas or precursor thereto. Exemplary bubbles include, for example, liposomes, micelles and the like. As discussed more fully hereinafter, the effectiveness of bubbles as contrast agents depends upon various factors, including, for example, the size and/or elasticity of the bubble. It has been noted that bubbles may emit a second harmonic frequency (i.e., when the bubbles are insonated at the 1x, or fundamental frequency they will reflect signals at not only the 1x frequency but also the 2x, or second harmonic frequency). Recently it has also been shown that solid tissues may also emit a 2x or second harmonic frequency. Cavitation imaging has also been demonstrated within tissues such as the liver via the Kupfer cells which phagocytose particles (viz gas filled microspheres).

In the prior art, bubbles have also been described as potentiators for ultrasonic hyperthermia and also as potentiators for sonothrombolysis. Second harmonic superimposition has also been described using two different frequencies of sound with a first 1x frequency and a second frequency ranging from 2x to 4x.

With respect to the effect of bubble size, the following discussion is provided. As known to the skilled artisan, the signal which is reflected off of a bubble is a function of the radius (r^6) of the bubble (Rayleigh Scatterer). Thus, in the frequency range of diagnostic ultrasound, a bubble having a diameter of 4 micrometer (μm) possesses about 64 times the scattering ability of a bubble having a diameter of 2 μm . Thus, generally speaking, the larger the bubble, the greater the reflected signal.

However, bubble size is limited by the diameter of capillaries through which the bubbles must pass. Generally, contrast agents which comprise bubbles having a diameter of greater than 10 μm can be dangerous since microvessels may be occluded. Accordingly, it is desired that greater than about 99% of the bubbles in a contrast agent have a diameter of less than 10 μm . Mean bubble diameter is important also, and should be greater than 1 μm , with greater than 2 μm being preferred. The volume weighted mean diameter of the bubbles should be about 7 to 10 micrometer.

The elasticity of bubbles is also important. This is because highly elastic bubbles can deform, as necessary, to "squeeze" through capillaries and/or to permit the flow of blood around the bubbles. This decreases the likelihood of occlusion. The effectiveness of a contrast agent which comprises bubbles is also dependent on the bubble

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concentration. Generally, the higher the bubble concentration, the greater the reflectivity of the contrast agent.

Another important characteristic which is related to the effectiveness of bubbles as contrast agents is bubble stability. As used herein, particularly with reference to gas-filled bubbles, "bubble stability" refers to the ability of bubbles to retain gas entrapped therein after exposure to a pressure greater than atmospheric pressure. To be effective as contrast agents, bubbles generally need to retain greater than 50% of entrapped gas after exposure to pressure of 300 millimeters (mm) of mercury (Hg) for about one minute. Particularly effective bubbles retain 75% of the entrapped gas after being exposed for one minute to a pressure of 300 mm Hg, with an entrapped gas content of 90% providing especially effective contrast agents. It is also highly desirable that, after release of the pressure, the bubbles return to their original size. This is referred to generally as "bubble resilience."

Bubbles which lack desirable stability provide poor contrast agents. If, for example, bubbles release the gas entrapped therein *in vivo*, reflectivity is diminished. Similarly, the size of bubbles which possess poor resilience will be decreased *in vivo*, also resulting in diminished reflectivity.

The stability of bubbles disclosed in the prior art is generally inadequate for use as contrast agents. For example, the prior art discloses bubbles, including gas-filled liposomes, which comprise lipid-containing walls or membranes. *See, e.g.*, Ryan et al., U.S. Patent Nos. 4,900,540 and 4,544,545; Tickner et al., U.S. Patent No. 4,276,885; Klaveness et al., WO 93/13809 and Schneider et al., EPO 0 554 213 and WO 91/15244. Lanza et al., WO 93/20802 discloses acoustically reflective oligolamellar liposomes, which are multilamellar liposomes with increased aqueous space between bilayers or have liposomes nested within bilayers in a nonconcentric fashion, and thus contain internally separated bilayers. Their use as ultrasonic contrast agents to enhance ultrasonic imaging, and in monitoring a drug delivered in a liposome administered to a patient, is also described. D'Arrigo, U.S. Patent Nos. 4,684,479 and 5,215,680 disclose gas-in-liquid emulsions and lipid-coated microbubbles, respectively.

Many of the bubbles disclosed in the prior art have undesirably poor stability. Thus, the prior art bubbles are more likely to rupture *in vivo* resulting, for

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example, in the untimely release of any therapeutic and/or diagnostic agent contained therein. Various studies have been conducted in an attempt to improve bubble stability. Such studies have included, for example, the preparation of bubbles in which the membranes or walls thereof comprise proteins, such as albumin, or materials which are 5 apparently strengthened via crosslinking. *See, e.g.*, Klaveness et al., WO 92/17212, in which there are disclosed bubbles which comprise proteins crosslinked with biodegradable crosslinking agents. A presentation was made by Moseley et al., at a 1991 Napa, California meeting of the Society for Magnetic Resonance in Medicine, which is summarized in an abstract entitled "Microbubbles: A Novel MR Susceptibility Contrast 10 Agent." The microbubbles described by Moseley et al. comprise air coated with a shell of human albumin. Alternatively, bubble membranes can comprise compounds which are not proteins but which are crosslinked with biocompatible compounds. *See, e.g.*, Klavness et al., WO 92/17436, WO 93/17718 and WO 92/21382.

Prior art techniques for stabilizing bubbles, including the use of proteins in 15 the outer membrane or crosslinking of the membrane components, suffer from various drawbacks. For example, the crosslinking described above generally involves the use of new materials, including crosslinked proteins or other compounds, for which the metabolic fate is unknown. In addition, crosslinking requires additional chemical process steps, including isolation and purification of the crosslinked compounds. Moreover, the use in 20 bubble membranes of proteins, such as albumin, and crosslinking of the bubble membrane components, can impart rigidity to the walls of the bubbles. This results in bubbles having reduced elasticity and, therefore, a decreased ability to deform and pass through capillaries. Thus, there is a greater likelihood of occlusion of vessels with prior art contrast agents that involve proteins and/or crosslinking.

25 Accordingly, new and/or better stabilized contrast agents and methods for providing same are needed. The present invention is directed to this, as well as other, important ends.

Summary of the Invention

The present invention is directed, in part, to ultrasound methods. 30 Specifically, in one embodiment, there is provided an ultrasound method comprising (i)

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administering to a patient a targeted composition which comprises a lipid, protein or polymer and a gas, in combination with a targeting ligand; and (ii) scanning the patient using ultrasound, wherein the scanning comprises exposing the patient to a first type of ultrasound energy and then interrogating the patient using a second type of ultrasound energy. In preferred embodiments, the lipids, proteins or polymers are in the form of vesicles. Also in preferred embodiments, the vesicles encapsulate the gas which is preferably a fluorinated gas selected from the group consisting of perfluorocarbons and sulfur hexafluoride. Yet also in preferred embodiments, the targeting ligand targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein GPIIbIIIa receptor.

Generally speaking, the present invention preferably involves the use of a narrow excitation frequency bandwidth and an inroad bandwidth sampling frequency with a digital multifrequency filter gate. Ultrasonic pulsing may be phase and temporally modulated over a range of frequencies. The methods of the invention may be utilized to increase the signal received from microbubbles while decreasing background tissue signals.

These and other aspects of the invention will become more apparent from the following detailed description.

Brief Description of the Drawings

For the purpose of illustrating embodiments of the invention, there is shown in the drawings forms which are presently preferred. It should be understood, however, that this invention is not limited to the precise arrangements and instrumentalities shown.

Figure 1 is a schematic representation of a system for the *in vitro* evaluation of contrast agents in accordance with embodiments of the present invention.

Figure 2 is a trace of ultrasound signal intensity of an *in vitro* blood clot before and after exposure to a contrast agent of the prior art.

Figure 3 is a trace of ultrasound signal intensity of an *in vitro* blood clot before and after exposure to a contrast agent in accordance with an embodiment of the present invention.

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Figure 4 is a trace of ultrasound signal intensity of an *in vitro* blood clot before and after exposure to a contrast agent in accordance with an alternate embodiment of the present invention.

Figure 5 is a diagrammatic representation of a thrombus in combination with a targeted vesicle composition. As depicted in Figure 5, there may be a large reflective acoustic interface presented by the vesicles on the surface of the thrombus and within the thrombus itself. The thrombus, particularly an acute thrombus, represents a porous matrix to which multiple vesicles may be bound. Because of the multiplicity of vesicles, the target-bound vesicles may represent a specular reflector much larger than the individual vesicles themselves.

Figure 6 depicts conventional gas filled vesicles in solution. The vesicles may be distributed relatively homogeneously within a liquid medium, for example, gas encapsulated lipid-coated vesicles (hereinafter sometimes referred to as "non-targeted vesicles") in the blood. As depicted in Figure 6, each vesicle generally represents a relatively small scatterer on the order of microns in diameter. This presents a different acoustic interface as presented by the targeted vesicles as shown in Figure 5. The targeted vesicles of Figure 5 may create a much more efficient ultrasonic scattering interface than the non-targeted vesicle composition of Figure 6.

Figure 7 shows the harmonic signatures of clot, clot in combination with non-targeted vesicle composition, and clot in combination with targeted vesicle composition.

Figure 8 is a diagram of subharmonic signals from the targeted vesicle composition bound to a blood clot when the clot is insonated at a frequency of 2.25 MHz at a peak acoustic pressure of 1.0 megapascals. As shown in Figure 8, the frequency and phase of the subharmonic signals may be predicted with respect to the fundamental insonating frequency.

Figure 9 demonstrates how a digital filter range gate may be varied with respect to the fundamental insonating frequency of the ultrasound. This composite diagram illustrates the range of subharmonic and harmonic frequencies of the signals from targeted vesicle composition bound to thrombus using an insonating frequency of 2.5 MHz. The peaks of each of the subharmonic and harmonic frequencies may be predicted

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knowing the center insonating frequency. Each range gate may be preferably positioned at about either m/n times x or $2(1+n)$ times x , where x is equal to the insonating frequency and m and n are integers not equal to each other describing the number respectively of the sub and ultra-harmonic signal. Each digital signal range gate may be centered at the
5 respective sub and harmonic frequency and extends over a bandwidth less than 1/2 the fundamental insonating frequency.

Figure 10 shows the improvement in detection or enhancement for different thrombi. As noted, the largest improvement in enhancement and detection may be for
10 hypoechoic thrombi. For hyperechoic thrombi, the improvement may be very slight. In an echogenic tissue, it is generally much more difficult to detect thrombus with conventional imaging.

Figure 11 shows *in vitro* insonation of thrombus. Comparison is shown using one frequency of insonation with using 2 different frequencies of insonation.
15 Signal-to-noise may be greatly amplified when two different frequencies are used.

Figure 12 illustrates phase modulation of ultrasound. In this case, a first 0.5 MHz pulse is administered and within 40 milliseconds a second pulse of 3.0 MHz is administered (180 degrees out of phase with the first pulse). Although a multifrequency insonation regime is depicted in this diagram, two pulses of the same frequency but varying phase and/or time delay may also be employed. The result may be decreased
20 background signal and improved signal-to-noise.

Figure 13 depicts the effects of sonothrombolysis of targeted microbubble agent using 20 kHz, 1.0 MHz and 20 kHz + 1.0 MHz ultrasound from actual experimental data. A hypothetical result is shown for 20 kHz + 1.0 MHz ultrasound when the pulsing and phase is modulated for maximal effect. For the same acoustic intensity level, 20 kHz
25 is more effective than 1.0 MHz and the effect of non-phase-non-pulse coordinated combination ultrasound is approximately additive. When the phase and pulsing and/or time delay is timed optimally, a synergistic effect may be obtained between the 20 kHz and 1.0 MHz ultrasound. This may result in increased sonothrombolysis.

Figure 14 is a schematic diagram of a device comprising one or more
30 frequency of sound phase and pulse modulation and a digital multi-range-gate filter.

Detailed Description of the Invention

As employed above and throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

- "Lipid" refers to a synthetic or naturally-occurring compound which is generally amphipathic and biocompatible. The lipids typically comprise a hydrophilic component and a hydrophobic component. Exemplary lipids include, for example, fatty acids, neutral fats, phosphatides, glycolipids, surface-active agents (surfactants), aliphatic alcohols, waxes, terpenes and steroids. In certain preferred embodiments, the lipids which may be incorporated in the compositions described herein contain no sulfhydryl groups or disulfide linkages.

"Lipid composition" refers to a composition which comprises a lipid compound, typically in an aqueous medium. Exemplary lipid compositions include suspensions, emulsions and vesicle compositions.

- "Lipid formulation" refers to a lipid composition which also comprises a bioactive agent.

"Vesicle" refers to a spherical entity which is generally characterized by the presence of one or more walls or membranes which form one or more internal voids. Vesicles may be formulated, for example, from lipids, including the various lipids described herein, proteinaceous materials, or polymeric materials, including natural, synthetic and semi-synthetic polymers. Preferred vesicles are those which comprise walls or membranes formulated from lipids. In these preferred vesicles, the lipids may be in the form of a monolayer or bilayer, and the mono- or bilayer lipids may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers may be concentric. Lipids may be used to form a unilamellar vesicle (comprised of one monolayer or bilayer), an oligolamellar vesicle (comprised of about two or about three monolayers or bilayers) or a multilamellar vesicle (comprised of more than about three monolayers or bilayers). Similarly, the vesicles prepared from proteins or polymers may comprise one or more concentric walls or membranes. The walls or membranes of vesicles prepared from proteins or polymers may be substantially solid (uniform), or they may be porous or semi-porous. In certain preferred embodiments, the vesicles contain no sulfhydryl groups or disulfide linkages. The vesicles described herein include such entities

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- commonly referred to as, for example, liposomes, micelles, bubbles, microbubbles, microspheres, lipid-, polymer- and/or protein-coated bubbles, microbubbles and/or microspheres, microballoons, aerogels, clathrate bound vesicles, and the like. The internal void of the vesicles may be filled with a liquid (including, for example, an aqueous liquid),
- 5 a gas, a gaseous precursor, and/or a solid or solute material, including, for example, a targeting ligand and/or a bioactive agent, as desired.

"Liposome" refers to a generally spherical cluster or aggregate of amphipathic compounds, including lipid compounds, typically in the form of one or more concentric layers, for example, monolayers and/or bilayers. They may also be referred to
10 herein as lipid vesicles. The liposomes may be formulated, for example, from ionic lipids and/or non-ionic lipids. Liposomes which are formulated from non-ionic lipids may also be referred to as "niosomes."

"Micelle" refers to colloidal entities formulated from lipids. In certain preferred embodiments, the micelles comprise a monolayer or hexagonal H₂ phase
15 configuration. In other preferred embodiments, the micelles may comprise a bilayer configuration.

"Aerogel" refers to generally spherical entities which are characterized by a plurality of small internal voids. The aerogels may be formulated from synthetic materials (for example, a foam prepared from baking resorcinol and formaldehyde), as well as
20 natural materials, such as polysaccharides or proteins.

"Clathrate" refers to a solid, semi-porous or porous particle which may be associated with vesicles. In preferred form, the clathrates may form a cage-like structure containing cavities which comprise the vesicles. One or more vesicles may be bound to the clathrate. A stabilizing material may, if desired, be associated with the clathrate to
25 promote the association of the vesicle with the clathrate. Suitable materials from which clathrates may be formulated include, for example, porous apatites, such as calcium hydroxyapatite, and precipitates of polymers and metal ions, such as alginic acid precipitated with calcium salts.

The vesicles of the present invention preferably contain a gas or gaseous precursor. "Gas filled vesicle" refers to vesicles in which there is encapsulated a gas.
30 "Gaseous precursor filled vesicle" refers to vesicles in which there is encapsulated a

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gaseous precursor. The vesicles may be minimally, partially or substantially completely filled with the gas and/or gaseous precursor. In certain preferred embodiments, the vesicles may be substantially or completely filled with the gas and/or gaseous precursor. The term "substantially", as used in reference to the gas and/or gaseous precursor filled vesicles, means that greater than about 50% of the internal void volume of the vesicle consists of a gas. Preferably, greater than about 60% of the internal void of the substantially filled vesicles consists of a gas, with greater than about 70% being more preferred. Even more preferably, greater than about 80% of the internal void of the substantially filled vesicles consists of a gas, with greater than about 90% being still more preferred. In particularly preferred embodiments, greater than about 95% of the internal void of the vesicles consists of a gas, with about 100% being especially preferred. Although not considered a preferred embodiment of the present invention, the vesicles may also contain, if desired, no or substantially no gas or gaseous precursor.

"Vesicle composition" refers to a composition, typically in an aqueous medium, which comprises vesicles.

"Vesicle formulation" refers to a vesicle composition which also comprises a bioactive agent. Suitable vesicles or vesicle species for use in vesicle formulations include, for example, gas filled vesicles and gaseous precursor filled vesicles.

"Emulsion" refers to a lipoidal mixture of two or more liquids and is generally in the form of a colloid. The lipids may be heterogeneously dispersed throughout the emulsion. Alternatively, the lipids may be aggregated in the form of, for example, clusters or layers, including mono- or bilayers.

"Suspension" refers to a mixture of finely divided liquid or solid particles floating in a liquid which can remain stable for extended periods of time.

"Hexagonal H II phase structure" refers to a generally tubular aggregation of lipids in liquid media, for example, aqueous media, in which the hydrophilic portion(s) of the lipids generally face inwardly in association with a liquid environment inside the tube. The hydrophobic portion(s) of the lipids generally radiate outwardly and the complex assumes the shape of a hexagonal tube. A plurality of tubes is generally packed together in the hexagonal phase structure.

"Patient" refers to animals, including mammals, preferably humans.

The phrases "internal region of a patient" and "region of interest" refer to the entire patient or to a particular area or portion of the patient. Internal regions of a patient and regions of interest may include, for example, areas being imaged with diagnostic imaging and/or areas being treated with a bioactive agent. Exemplary of such areas include, for example, the heart region, including myocardial tissue, as well as other bodily tissues, including the vasculature and circulatory system and cancerous tissue. The phrase "vasculature," as used herein, denotes the blood vessels in the body or in an organ or part of the body.

"Bioactive agent" refers to a substance which may be used in connection with an application that is therapeutic or diagnostic in nature, such as in methods for diagnosing the presence or absence of a disease in a patient and/or in methods for the treatment of disease in a patient. As used herein, "bioactive agent" refers also to substances which are capable of exerting a biological effect *in vitro* and/or *in vivo*. The bioactive agents may be neutral or positively or negatively charged. Examples of suitable bioactive agents include diagnostic agents, pharmaceuticals, drugs, synthetic organic molecules, proteins, peptides, vitamins, steroids and genetic material, including nucleosides, nucleotides and polynucleotides.

"Diagnostic agent" refers to any agent which may be used in connection with methods for imaging an internal region of a patient and/or diagnosing the presence or absence of a disease in a patient. Exemplary diagnostic agents include, for example, contrast agents for use in connection with ultrasound, magnetic resonance imaging or computed tomography of a patient including, for example, the lipid and/or vesicle compositions described herein.

"Polymer", as used herein, refers to molecules formed from the chemical union of two or more repeating units. Accordingly, included within the term "polymer" may be, for example, dimers, trimers and oligomers. The polymer may be synthetic, naturally-occurring or semisynthetic. In preferred form, the term "polymer" refers to molecules which comprise 10 or more repeating units. In certain preferred embodiments, the polymers which may be incorporated in the compositions described herein contain no sulphhydryl groups or disulfide linkages.

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"Thickening agent" refers to any of a variety of generally hydrophilic materials which, when incorporated in the lipid and/or vesicle compositions described herein, may act as viscosity modifying agents, emulsifying and/or solubilizing agents, suspending agents, and tonicity raising agents. It is contemplated that the thickening agents may be capable of aiding in maintaining the stability of the compositions due to such properties.

"Dispersing agent" refers to a surface-active agent which, when added to a suspending medium of colloidal particles, including, for example, certain of the lipid and/or vesicle compositions described herein, may promote uniform separation of particles. In certain preferred embodiments, the dispersing agent may comprise a polymeric siloxane compound.

"Genetic material" refers generally to nucleotides and polynucleotides, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The genetic material may be made by synthetic chemical methodology known to one of ordinary skill in the art, or by the use of recombinant technology, or by a combination of the two. The DNA and RNA may optionally comprise unnatural nucleotides and may be single or double stranded. "Genetic material" refers also to sense and anti-sense DNA and RNA, that is, a nucleotide sequence which is complementary to a specific sequence of nucleotides in DNA and/or RNA.

"Pharmaceutical" or "drug" refers to any therapeutic or prophylactic agent which may be used in the treatment (including the prevention, diagnosis, alleviation, or cure) of a malady, affliction, disease or injury in a patient. Therapeutically useful peptides, polypeptides and polynucleotides may be included within the meaning of the term pharmaceutical or drug.

"Stabilizing material" refers to any material which is capable of improving the stability of compositions as described herein including, for example, emulsions, suspensions, dispersions and vesicle compositions. The improved stability involves, for example, the maintenance of a relatively balanced condition, and may be exemplified, for example, by increased resistance against destruction, decomposition, degradation and the like. In the case of preferred embodiments involving vesicles, especially gas filled vesicles, the stabilizing compounds may serve to improve the stability of the vesicles, for

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example, by minimizing or substantially (including completely) preventing the escape of gas entrapped within vesicles which may occur, for example, from rupture and/or coalescence of vesicles. The term "substantially", as used in reference to the prevention of the escape of entrapped gas, means that greater than about 50% of the gas is maintained

5 entrapped. Preferably, greater than about 60% of the gas is maintained entrapped, with greater than about 70% being more preferred. Even more preferably, greater than about 80% of the gas is maintained entrapped, with greater than about 90% being still more preferred. In particularly preferred embodiments, greater than about 95% of the gas is maintained entrapped. If desired, the gas may be completely maintained entrapped (i.e.,

10 about 100% of the gas is maintained entrapped). The stabilizing compounds may comprise discrete, individual compounds (monomers), or may comprise polymers. In the case of preferred embodiments involving lipids, the stabilizing materials may be associated covalently and/or non-covalently with the lipid compounds. Broadly speaking, the stabilizing compounds may comprise, for example, surfactants, film-forming materials,

15 membranes and/or membrane forming materials. Exemplary stabilizing compounds which may be employed in the methods and compositions of the present invention include lipids, proteins and polymers. Encompassed also in the definition of "stabilizing material" are certain of the present bioactive agents. The stabilizing material may be neutral or positively or negatively charged. Preferred among the neutral stabilizing materials are

20 polar materials. In certain embodiments, the stabilizing compounds may be substantially (including completely) crosslinked. The terms "crosslink", "crosslinked" and "crosslinking", as used herein, generally refers to the linking of two or more stabilizing compounds, including lipid, protein and polymer stabilizing compounds, by one or more bridges. The bridges, which may be composed of one or more elements, groups or

25 compounds, generally serve to join an atom from a first stabilizing compound molecule to an atom of a second stabilizing molecule. The crosslink bridges may involve covalent and/or non-covalent associations. Any of a variety of elements, groups and/or compounds may form the bridges in the crosslinks, and the stabilizing compounds may be crosslinked naturally or through synthetic means. For example, crosslinking may occur in nature in

30 materials formulated from peptide chains which are joined by disulfide bonds of cystine residues, as in keratins, insulin, and other proteins. Alternatively, crosslinking may be

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effected by suitable chemical modification, such as, for example, by combining a compound, such as a stabilizing material, and a chemical substance that may serve as a crosslinking agent, which are caused to react, for example, by exposure to heat, high-energy radiation, ultrasonic radiation, and the like. Examples include, for example,

5 crosslinking with sulfur which may be present, for example, as sulfhydryl groups in cysteine residues, to provide disulfide linkages, crosslinking with organic peroxides, crosslinking of unsaturated materials by means of high-energy radiation, crosslinking with dimethylol carbamate, and the like. The term "substantially", as used in reference to crosslinked stabilizing compounds, means that greater than about 50% of the stabilizing

10 compounds contain crosslinking bridges. In certain embodiments, preferably greater than about 60% of the crosslinked stabilizing compounds contain crosslinking bridges, with greater than about 70% being more preferred. Even more preferably, greater than about 80% of the crosslinked stabilizing compounds contain crosslinking bridges, with greater than about 90% being still more preferred. In certain particularly preferred embodiments,

15 greater than about 95% of the crosslinked stabilizing compounds contain crosslinking bridges. If desired, the substantially crosslinked stabilizing compounds may be completely crosslinked (i.e., about 100% of the crosslinked stabilizing compounds contain crosslinking bridges). In the most preferred embodiments, the stabilizing compounds may be substantially (including completely) non-crosslinked. The term "substantially", as used

20 in reference to non-crosslinked stabilizing compounds, means that greater than about 50% of the stabilizing compounds are devoid of crosslinking bridges. Preferably, greater than about 60% of the stabilizing compounds are devoid of crosslinking bridges, with greater than about 70% being more preferred. Even more preferably, greater than about 80% of the stabilizing compounds are devoid of crosslinking bridges, with greater than about 90%

25 being still more preferred. In particularly preferred embodiments, greater than about 95% of the stabilizing compounds are devoid of crosslinking bridges. If desired, the substantially non-crosslinked stabilizing compounds may be completely non-crosslinked (i.e., about 100% of the stabilizing compounds are devoid of crosslinking bridges).

"Vesicle stability" refers to the ability of gas-filled vesicles to retain the gas entrapped therein after being exposed, for about one minute, to a pressure of about 300 mm Hg. Vesicle stability is measured in percent (%), this being the fraction of the amount

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of gas which is originally entrapped in the vesicle and which is retained after release of the pressure. Vesicle stability includes reference also to "vesicle resilience" which refers to the ability of a vesicle to return to its original size after release of the pressure.

"Covalent association" refers to an intermolecular association or bond
5 which involves the sharing of electrons in the bonding orbitals of two atoms.

"Non-covalent association" refers to intermolecular interaction among two or more separate molecules which does not involve a covalent bond. Intermolecular interaction is dependent upon a variety of factors, including, for example, the polarity of the involved molecules, the charge (positive or negative), if any, of the involved
10 molecules, and the like. Non-covalent associations are preferably selected from the group consisting of ionic interaction, dipole-dipole interaction and van der Waal's forces and combinations thereof.

"Ionic interaction" or "electrostatic interaction" refers to intermolecular interaction among two or more molecules, each of which is positively or negatively charged.
15 Thus, for example, "ionic interaction" or "electrostatic interaction" refers to the attraction between a first, positively charged molecule and a second, negatively charged molecule. Exemplary ionic or electrostatic interactions include, for example, the attraction between a negatively charged stabilizing material, for example, genetic material, and a positively charged lipid, for example, a cationic lipid, such as lauryltrimethylammonium
20 bromide.

"Dipole-dipole interaction" refers generally to the attraction which can occur among two or more polar molecules. Thus, "dipole-dipole interaction" refers to the attraction of the uncharged, partial positive end of a first polar molecule, commonly designated as δ^+ , to the uncharged, partial negative end of a second polar molecule,
25 commonly designated as δ^- . Dipole-dipole interactions are exemplified, for example, by the attraction between the electropositive head group, for example, the choline head group, of phosphatidylcholine and an electronegative atom, for example, a heteroatom, such as oxygen, nitrogen or sulphur, which is present in a stabilizing material, such as a polysaccharide. "Dipole-dipole interaction" refers also to intermolecular hydrogen
30 bonding in which a hydrogen atom serves as a bridge between electronegative atoms on

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separate molecules and in which a hydrogen atom is held to a first molecule by a covalent bond and to a second molecule by electrostatic forces.

- "Van der Waal's forces" refers to the attractive forces between non-polar molecules that are accounted for by quantum mechanics. Van der Waal's forces are
- 5 generally associated with momentary dipole moments which are induced by neighboring molecules and which involve changes in electron distribution.

"Hydrogen bond" refers to an attractive force, or bridge, which may occur between a hydrogen atom which is bonded covalently to an electronegative atom, for example, oxygen, sulfur, nitrogen, and the like, and another electronegative atom. The

10 hydrogen bond may occur between a hydrogen atom in a first molecule and an electronegative atom in a second molecule (intermolecular hydrogen bonding). Also, the hydrogen bond may occur between a hydrogen atom and an electronegative atom which are both contained in a single molecule (intramolecular hydrogen bonding).

"Hydrophilic interaction" refers to molecules or portions of molecules

15 which may substantially bind with, absorb and/or dissolve in water. This may result in swelling and/or the formation of reversible gels.

"Hydrophobic interaction" refers to molecules or portions of molecules which do not substantially bind with, absorb and/or dissolve in water.

"Biocompatible" refers to materials which are generally not injurious to

20 biological functions and which will not result in any degree of unacceptable toxicity, including allergenic responses and disease states.

"In combination with" refers, in certain embodiments, to the incorporation of a targeting ligand in a composition of the present invention, including lipid compositions and vesicle compositions. "In combination with" may refer also to the

25 incorporation of a bioactive agent in a composition of the present invention, including lipid compositions and vesicle compositions. The bioactive agent and/or targeting ligand may be combined with the present compositions in any of a variety of ways. If desired, the bioactive agent and/or targeting ligand may be associated covalently with one or more components of the present compositions such as, for example, lipid compounds, proteins,

30 polymers and/or vesicles or other optional stabilizing materials. Also, if desired, there may be substantially no covalent association of the bioactive agent and/or targeting ligand

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with the other components of the present compositions such as, for example, the lipid compounds, proteins, polymers and/or vesicles or other optional stabilizing materials. The term "substantially no", as used herein in reference to the lack of covalent association of bioactive agent and/or targeting ligand with other components of the compositions such as,

5 for example, lipid compounds, proteins, polymers and/or vesicles, may mean that less than about 50% such as, for example, from about 0% to less than about 50% (and all specific percentages and combinations and subcombinations of ranges of percentages therein) of the bioactive agent and/or targeting ligand may be covalently associated with other components of the compositions. Preferably, less than about 40% of the bioactive

10 agent and/or targeting ligand may be covalently associated with other components of the compositions, with less than about 30% being more preferred. Even more preferably, less than about 20% of the bioactive agent and/or targeting ligand may be covalently associated with other components of the compositions, with less than about 10% being yet more preferred. In still more preferred embodiments, there may be completely no (*i.e.*, 0%)

15 covalent association of the bioactive agent and/or targeting ligand with other components of the compositions.

Bioactive agent and/or targeting ligand which have substantially no covalent association with other components of the compositions may sometimes be referred to herein as "unbound" or "free" bioactive agent and/or targeting ligand. In such 20 compositions, the bioactive agent and/or targeting ligand may, if desired, be associated non-covalently with other components of the compositions such as, for example, the lipid compounds, proteins, polymers and/or vesicles or other optional stabilizing materials. In addition, if desired, there may be substantially no non-covalent association of the unbound or free bioactive agent and/or targeting ligand with other components of the compositions 25 including, for example, lipid compounds, proteins, polymers and/or vesicles. The term "substantially no", as used herein in reference to the lack of non-covalent association of bioactive agent and/or targeting ligand with other components of the compositions such as, for example, lipid compounds, proteins, polymers and/or vesicles, may mean that less than about 50% such as, for example, from about 0% to less than about 50% (and all specific percentages and combinations and subcombinations of ranges of percentages 30 therein) of the unbound or free bioactive agent and/or targeting ligand may be associated

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non-covalently with other components of the compositions. Preferably, less than about 40% of the unbound or free bioactive agent and/or targeting ligand may be associated non-covalently with other components of the compositions, with less than about 30% being more preferred. Even more preferably, less than about 20% of the unbound or free

5 bioactive agent and/or targeting ligand may be associated non-covalently with other components of the compositions, with less than about 10% being yet more preferred. In still more preferred embodiments, there may be completely no (*i.e.*, 0%) non-covalent association of the unbound or free bioactive agent and/or targeting ligand with other components of the compositions.

10 In the case of vesicle compositions, the bioactive agent and/or targeting ligand may be entrapped within the internal void of the vesicle. The bioactive agent and/or targeting ligand may also be integrated within the layer(s) or wall(s) of the vesicle, for example, by being interspersed among lipids which are contained within the vesicle layer(s) or wall(s). In addition, it is contemplated that the bioactive agent and/or targeting

15 ligand may be located on the surface of a vesicle. In any case, the bioactive agent and/or targeting ligand may interact chemically with the walls of the vesicles, including, for example, the inner and/or outer surfaces of the vesicle and may remain substantially adhered thereto. Such interaction may take the form of, for example, covalent association or non-covalent association. In certain embodiments, the interaction may result in the

20 stabilization of the vesicle.

"Targeting ligand" refers to any material or substance which may promote targeting of tissues and/or receptors *in vivo* with the compositions of the present invention. The targeting ligand may be synthetic, semi-synthetic, or naturally-occurring. Materials or substances which may serve as targeting ligands include, for example, proteins, including antibodies, glycoproteins and lectins, peptides, polypeptides, saccharides, including mono- and polysaccharides, vitamins, steroids, steroid analogs, hormones, cofactors, bioactive agents, and genetic material, including nucleosides, nucleotides and polynucleotides.

A "precursor" to a targeting ligand refers to any material or substance which may be converted to a targeting ligand. Such conversion may involve, for example, anchoring a precursor to a targeting ligand. Exemplary targeting precursor moieties

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include maleimide groups, disulfide groups, such as ortho-pyridyl disulfide, vinylsulfone groups, azide groups, and α -iodo acetyl groups.

"Peptide" refers to a nitrogenous compound which may contain from about 2 to about 100 amino acid residues. In certain preferred embodiments, the peptides which
5 may be incorporated in the compositions described herein contain no sulphydryl groups or disulfide linkages.

"Protein" refers to a nitrogenous compound which may contain more than about 100 amino acid residues. In certain preferred embodiments, the proteins which may be incorporated in the compositions described herein contain no sulphydryl groups or
10 disulfide linkages.

"Coat" or "coating" refers to the interaction of the stabilizing material with the lipid and/or vesicles and may involve covalent and/or non-covalent association.

"Tissue" refers generally to specialized cells which may perform a particular function. It should be understood that the term "tissue," as used herein, may
15 refer to an individual cell or a plurality or aggregate of cells, for example, membranes or organs. The term "tissue" also includes reference to an abnormal cell or a plurality of abnormal cells. Exemplary tissues include, for example, myocardial tissue (also referred to as heart tissue or myocardium), including myocardial cells and cardiomyocytes, membranous tissues, including endothelium and epithelium, laminae, connective tissue,
20 including interstitial tissue, and tumors.

"Receptor" refers to a molecular structure within a cell or on the surface of the cell which is generally characterized by the selective binding of a specific substance. Exemplary receptors include, for example, cell-surface receptors for peptide hormones, neurotransmitters, antigens, complement fragments, and immunoglobulins and
25 cytoplasmic receptors for steroid hormones. An exemplary receptor within the context of the present invention is the glycoprotein GPIIbIIIa, which is a platelet integrin.

"Endothelial cells" or "endothelium" refers to an aggregate of cells and/or tissue which may be normal and/or diseased and which may comprise a single layer of flattened transparent endothelial cells that may be joined edge to edge or in an overlapping fashion to form a membrane. Endothelial cells are found on the free surfaces of the serous membranes, as part of the lining membrane of the heart, blood vessels, and lymphatics, on

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the surface of the brain and spinal cord, and in the anterior chamber of the eye.

Endothelium originates from the embryonic mesoblast and includes heart tissue, including infarcted heart tissue, cardiovasculature, the peripheral vasculature, such as arteries, veins, and capillaries (the location of which is noted as peripheral to the heart), blood clots and

- 5 the region surrounding atherosclerotic plaque.

"Epithelial cells" or "epithelium" refers to an aggregate of cells and/or tissue which may be normal and/or diseased and which may comprise one or more layers of cells that may be united together by an interstitial cementitious substance supported on a basement-membrane. Epithelium may be classified into various classes, including, for 10 example, a single layer of cells (simple epithelium); more than a single layer of cells (stratified epithelium); and about three or four layers of cells that are fitted together substantially without the appearance of stratification. The different forms of simple epithelium are usually referred to as squamous, pavement, columnar, glandular, spheroidal and/or ciliated. Epithelium originates from the embryonic epiblast or hypoblast.

15 Epithelium includes heart tissue, including infarcted heart tissue, cardiovasculature, the peripheral vasculature, such as arteries, veins, and capillaries, blood clots and the region surrounding atherosclerotic plaque.

"Myocardial" refers generally to heart tissue, including cardiomyocyte, myocardial, endocardial and epicardial cells. The term "myocardial" includes reference to 20 infarcted heart tissue, the cardiovasculature, the peripheral vasculature, such as arteries, veins, and capillaries (the location of which is noted as peripheral to the heart), blood clots, thrombi, and the region surrounding atherosclerotic plaque.

"Tumor cells" or "tumor" refers to an aggregate of abnormal cells and/or tissue which may be associated with diseased states that are characterized by uncontrolled 25 cell proliferation. The disease states may involve a variety of cell types, including, for example, endothelial, epithelial and myocardial cells. Included among the disease states are neoplasms, cancer, leukemia and restenosis injuries.

"Alkyl" refers to an aliphatic hydrocarbon group which may be straight, branched or cyclic having 1 to about 50 carbon atoms in the chain, and all combinations 30 and subcombinations of ranges therein. "Lower alkyl" refers to an alkyl group having 1 to about 8 carbon atoms. "Higher alkyl" refers to an alkyl group having about 10 to about 20

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carbon atoms. The alkyl group may be optionally substituted with one or more alkyl group substituents which may be the same or different, where "alkyl group substituent" includes halo, aryl, hydroxy, alkoxy, aryloxy, alkyloxy, alkylthio, arylthio, aralkyloxy, aralkylthio, carboxy alkoxy carbonyl, oxo and cycloalkyl. There may be optionally inserted along the alkyl group one or more oxygen, sulphur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is lower alkyl. "Branched" refers to an alkyl group in which a lower alkyl group, such as methyl, ethyl or propyl, is attached to a linear alkyl chain. Exemplary alkyl groups include methyl, ethyl, *i*-propyl, *n*-butyl, *t*-butyl, *n*-pentyl, heptyl, octyl, decyl, dodecyl, tridecyl, tetradecyl, pentadecyl and hexadecyl. Preferred alkyl groups include the lower alkyl groups of 1 to about 4 carbons and the higher alkyl groups of about 12 to about 16 carbons. Preferred alkyl groups include also alkyl groups which are substituted with one or more halo atoms. Fluoroalkyl groups are preferred among the halo-substituted alkyl groups, including, for example, fluoroalkyl groups of the formula $\text{CF}_3(\text{CF}_2)_n(\text{CH}_2)_m-$, wherein each of m and n is independently an integer from 0 to about 22. Exemplary fluoroalkyl groups include perfluoromethyl, perfluoroethyl, perfluoropropyl, perfluorobutyl, perfluorocyclobutyl, perfluoropentyl, perfluorohexyl, perfluoroheptyl, perfluoroctyl, perfluorononyl, perfluorodecyl, perfluoroundecyl and perfluorododecyl. Exemplary cyclic hydrocarbon groups (that is, cycloalkyl groups) include, for example, cyclopentyl, cyclohexyl and cycloheptyl groups. Exemplary cyclic hydrocarbon groups also include cycloalkenyl groups such as, for example, cyclopentenyl and cyclohexenyl, as well as hydrocarbon groups comprising fused cycloalkyl and/or cycloalkenyl groups including for example, steroid groups, such as cholesterol.

"Alkenyl" refers to an alkyl group containing at least one carbon-carbon double bond. Exemplary alkenyl groups include, for example, vinyl, allyl, butenyl, pentenyl, decenyl and dodecenyl groups.

"Alkoxy" refers to an alkyl-O- group where alkyl is as previously described. Exemplary alkoxy groups include, for example, methoxy, ethoxy, propoxy, butoxy and heptoxy.

"Alkylene" refers to a straight or branched bivalent aliphatic hydrocarbon group having from 1 to about 30 carbon atoms. The alkylene group may be straight,

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branched or cyclic. The alkylene group may be also optionally unsaturated and/or substituted with one or more "alkyl group substituents." There may be optionally inserted along the alkylene group one or more oxygen, sulphur or substituted or unsubstituted nitrogen atoms, wherein the nitrogen substituent is alkyl as previously described.

- 5 Exemplary alkylene groups include methylene (-CH₂-), ethylene (-CH₂CH₂-), propylene (-CH₂)₃-), cyclohexylene (-C₆H₁₀-), -CH=CH-CH=CH-, -CH=CH-CH₂-, -(CF₂)_n(CH₂)_m-, wherein n is an integer from about 1 to about 22 and m is an integer from 0 to about 22, -(CH₂)_n-N(R)-(CH₂)_m-, wherein each of m and n is independently an integer from 0 to about 30 and R is hydrogen or alkyl, methylenedioxy (-O-CH₂-O-) and ethylenedioxy
- 10 -O-(CH₂)₂-O-). It is preferred that the alkylene group has about 1 to about 3 carbon atoms.

The present invention is directed, in part, to lipid and/or vesicle compositions. Embodiments are provided which comprise a lipid compositions comprising a lipid, a targeting ligand which may target tissues, cells and/or receptors *in vivo* and which may be attached to the lipid via a linking group, and a gas or gaseous precursor. Embodiments are also provided herein which comprise vesicle compositions comprising, in an aqueous carrier, vesicles comprising lipids, proteins or polymers, a targeting ligand which may target tissues, cells and/or receptors *in vivo*, and a gas or gaseous precursor. In these latter embodiments, the targeting ligand may be attached to the vesicles, including the lipids, proteins or polymers from which the vesicles are formulated, via a linking group. In connection with lipid compositions, and especially lipid compositions in the form of vesicle compositions, it may be advantageous to prepare the lipid compositions at a temperature below the gel to liquid crystalline phase transition temperature of the involved lipids. This phase transition temperature is the temperature at which a lipid bilayer will convert from a gel state to a liquid crystalline state. See, for example, Chapman *et al.*, *J. Biol. Chem.* 1974 249, 2512-2521.

It is generally believed that vesicles which are prepared from lipids that possess higher gel state to liquid crystalline state phase transition temperatures tend to have enhanced impermeability at any given temperature. See Derek Marsh, *CRC Handbook of Lipid Bilayers* (CRC Press, Boca Raton, FL 1990), at p. 139 for main chain melting transitions of saturated diacyl-sn-glycero-3-phosphocholines. The gel state to

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liquid crystalline state phase transition temperatures of various lipids will be readily apparent to those skilled in the art and are described, for example, in Gregoriadis, ed., *Liposome Technology*, Vol. I, 1-18 (CRC Press, 1984). The following table lists some of the representative lipids and their phase transition temperatures.

5

TABLE 1
**Saturated Diacyl-sn-Glycero-3-Phosphocholines:
Main Chain Melting Transition Temperatures**

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Number of Carbons in Acyl Chains	Main Phase Transition Temperature (° C)
1,2-(12:0)	-1.0
1,2-(13:0)	13.7
1,2-(14:0)	23.5
1,2-(15:0)	34.5
1,2-(16:0)	41.4
1,2-(17:0)	48.2
1,2-(18:0)	55.1
1,2-(19:0)	61.8
1,2-(20:0)	64.5
1,2-(21:0)	71.1
1,2-(22:0)	74.0
1,2-(23:0)	79.5

TABLE 1	
Saturated Diacyl-sn-Glycero-3-Phosphocholines: Main Chain Melting Transition Temperatures	
Number of Carbons in Acyl Chains	Main Phase Transition Temperature (° C)
1,2-(24:0)	80.1

See, for example, Derek Marsh, *CRC Handbook of Lipid Bilayers*, p. 139 (CRC Press, Boca Raton, FL 1990).

It may be possible to enhance the stability of vesicles by incorporating in 5 the present lipid and/or vesicle compositions at least a minor amount, for example, about 1 to about 10 mole percent, based on the total amount of lipid employed, of a negatively charged lipid. Suitable negatively charged lipids include, for example, phosphatidylserine, phosphatidic acid, and fatty acids. Without intending to be bound by any theory or theories of operation, it is contemplated that such negatively charged lipids provide added 10 stability by counteracting the tendency of vesicles to rupture by fusing together. Thus, the negatively charged lipids may act to establish a uniform negatively charged layer on the outer surface of the vesicle, which will be repulsed by a similarly charged outer layer on other vesicles which are proximate thereto. In this way, the vesicles may be less prone to come into touching proximity with each other, which may lead to a rupture of the 15 membrane or skin of the respective vesicles and consolidation of the contacting vesicles into a single, larger vesicle. A continuation of this process of consolidation will, of course, lead to significant degradation of the vesicles.

The lipid materials used, especially in connection with vesicle compositions, are also preferably flexible. This means, in the context of the present 20 invention, that the vesicles can alter their shape, for example, to pass through an opening having a diameter that is smaller than the diameter of the vesicle.

A wide variety of lipids are believed to be suitable for incorporation in the lipid compositions. With particular reference to vesicle compositions, for example,

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micelles and/or liposomes, any of the materials or combinations thereof which are known to those skilled in the art as suitable for their preparation may be used. The lipids used may be of either natural, synthetic or semi-synthetic origin. As noted above, suitable lipids generally include, for example, fatty acids, neutral fats, phosphatides, glycolipids, 5 aliphatic alcohols and waxes, terpenes, sesquiterpenes, and steroids.

Exemplary lipids which may be used to prepare the present lipid compositions included, for example, fatty acids, lysolipids, including lysophospholipids, phosphocholines, such as those associated with platelet activation factors (PAF) (Avanti Polar Lipids, Alabaster, AL), including 1-alkyl-2-acetyl-sn-glycero 3-phosphocholines, 10 and 1-alkyl-2-hydroxy-sn-glycero 3-phosphocholines, which target blood clots; phosphatidylcholine with both saturated and unsaturated lipids, including dioleoylphosphatidylcholine; dimyristoylphosphatidylcholine; dipentadecanoyl-phosphatidylcholine; dilauroylphosphatidylcholine; dipalmitoylphosphatidylcholine (DPPC); distearoylphosphatidylcholine (DSPC); and diarachidonylphosphatidylcholine 15 (DAPC); phosphatidylethanolamines, such as dioleoylphosphatidylethanolamine, dipalmitoylphosphatidylethanolamine (DPPE) and distearoylphosphatidylethanolamine (DSPE); phosphatidylserine; phosphatidylglycerols, including distearoylphosphatidylglycerol (DSPG) and dipalmitoyl-glycerolsuccinate (DPGS); phosphatidylinositol; sphingolipids such as sphingomyelin; sphingosines; glycolipids such as ganglioside GM1 20 and GM2; glucolipids; sulfatides; glycosphingolipids; phosphatidic acids, such as dipalmitoylphosphatidic acid (DPPA) and distearoylphosphatidic acid (DSPA); palmitic acid; stearic acid; arachidonic acid; oleic acid; lipids bearing polymers, such as chitin, hyaluronic acid, polyvinylpyrrolidone or polyethylene glycol (PEG), also referred to herein as "pegylated lipids", with preferred lipids bearing polymers including DPPE-PEG 25 (DPPE-PEG), which refers to the lipid DPPE having a PEG polymer attached thereto, including, for example, DPPE-PEG5000, which refers to DPPE having attached thereto a PEG polymer having a mean average molecular weight of about 5000; lipids bearing sulfonated mono-, di-, oligo- or polysaccharides; steroids, such as for example, cholesterol, cholesterol sulfate, cholesterol hemisuccinate and cholesterol amines; tocopherol 30 hemisuccinate; lipids with ether and ester-linked fatty acids; polymerized lipids (a wide variety of which are well known in the art); diacetyl phosphate; dicetyl phosphate;

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- stearylamine; cardiolipin; phospholipids with short chain fatty acids of about 6 to about 8 carbons in length; synthetic phospholipids with asymmetric acyl chains, such as, for example, one acyl chain of about 6 carbons and another acyl chain of about 12 carbons; ceramides; non-ionic liposomes including niosomes such as polyoxyalkylene (e.g.,
- 5 polyoxyethylene) fatty acid esters, polyoxyalkylene (e.g., polyoxyethylene) fatty alcohols, polyoxyalkylene (e.g., polyoxyethylene) fatty alcohol ethers, polyoxyalkylene (e.g., polyoxyethylene) sorbitan fatty acid esters (such as, for example, the class of compounds referred to as TWEEN®, including, for example, TWEEN® 20, TWEEN® 40 and TWEEN® 80, commercially available from ICI Americas, Inc., Wilmington, DE), glycerol
- 10 polyethylene glycol oxystearate, glycerol polyethylene glycol ricinoleate, alkyloxylated (e.g., ethoxylated) soybean sterols, alkyloxylated (e.g., ethoxylated) castor oil, polyoxyethylene-polyoxypropylene polymers, and polyoxyalkylene (e.g., polyoxyethylene) fatty acid stearates; sterol aliphatic acid esters including cholesterol sulfate, cholesterol butyrate, cholesterol isobutyrate, cholesterol palmitate, cholesterol
- 15 stearate, lanosterol acetate, ergosterol palmitate, and phytosterol n-butyrate; sterol esters of sugar acids including cholesterol glucuronide, lanosterol glucuronide, 7-dehydro-cholesterol glucuronide, ergosterol glucuronide, cholesterol gluconate, lanosterol gluconate, and ergosterol gluconate; esters of sugar acids and alcohols including lauryl glucuronide, stearoyl glucuronide, myristoyl glucuronide, lauryl gluconate, myristoyl
- 20 gluconate, and stearoyl gluconate; esters of sugars and aliphatic acids including sucrose laurate, fructose laurate, sucrose palmitate, sucrose stearate, glucuronic acid, gluconic acid and polyuronic acid; saponins including sarsasapogenin, smilagenin, hederagenin, oleanolic acid, and digitoxigenin; glycerol dilaurate, glycerol trilaurate, glycerol dipalmitate, glycerol and glycerol esters including glycerol tripalmitate, glycerol
- 25 distearate, glycerol tristearate, glycerol dimyristate, glycerol trimyristate; long chain alcohols including n-decyl alcohol, lauryl alcohol, myristyl alcohol, cetyl alcohol, and n-octadecyl alcohol; 6-(5-cholest-3β-yloxy)-1-thio-β-D-galactopyranoside; digalactosyldiglyceride; 6-(5-cholest-3β-yloxy)-hexyl-6-amino-6-deoxy-1-thio-β-D-galactopyranoside; 6-(5-cholest-3β-yloxy)hexyl-6-amino-6-deoxyl-1-thio-α-D-
- 30 mannopyranoside; 12-(((7'-diethylamino-coumarin-3-yl)-carbonyl)methylamino)-octadecanoic acid; N-[12-(((7'-diethylamino-coumarin-3-yl)-carbonyl)methylamino)-

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octadecanoyl]-2-aminopalmitic acid; cholesteryl(4'-trimethyl-ammonio)butanoate; N-succinyl dioleoylphosphatidylethanolamine; 1,2-dioleoyl-sn-glycerol; 1,2-dipalmitoyl-sn-3-succinylglycerol; 1,3-dipalmitoyl-2-succinylglycerol; 1-hexadecyl-2-palmitoylglycerophosphoethanolamine and palmitoylhomocysteine, and/or any 5 combinations thereof. In preferred embodiments, the stabilizing materials comprise phospholipids, including one or more of DPPC, DPPE, DPPA, DSPC, DSPE, DSPG, and DAPC.

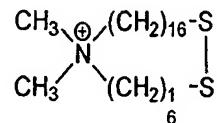
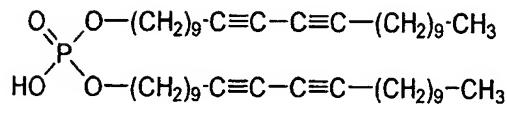
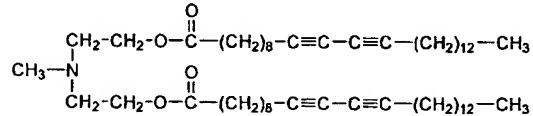
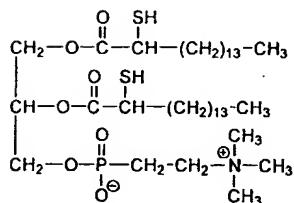
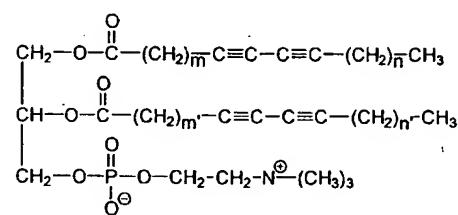
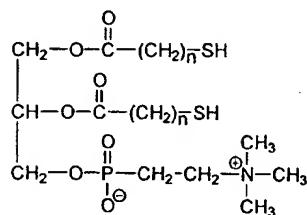
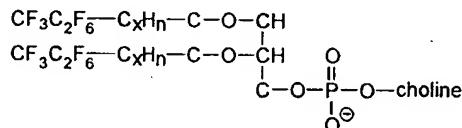
Examples of suitable fluorinated lipids include but are not limited to compounds of the formula:

- 10 $C_nF_{2n+1}(CH_2)_mC(O)OOP(OO')O(CH_2)_wN^+(CH_3)_3C_nF_{2n+1}(CH_2)_mC(O)O$
wherein m is 0 to about 18, n is 1 to about 12; and w is 1 to about 8. Examples of and methods for the synthesis of these, as well as other fluorinated lipids useful in the present invention, are set forth in U.S. application Serial No. 08/465,868, filed June 6, 1995, Reiss et al. U.S. Patent No. 5,344,930, Frezard, F., et al., *Biochem Biophys Acta*, 1192:61-70 (1994), and Frezard, F., et al., *Art. Cells Blood Subs and Immob Biotech.*, 22:1403-1408 (1994), the disclosures of each of which are incorporated herein by reference in their entirety. One specific example of a difluoroacyl glycerylphosphatidyl-choline, nonafluorinated diacyl glycerylphosphatidylcholine, is represented by compound A, below. One skilled in the art will appreciate that analogous fluorinated derivatives of other 15 common phospholipids (diacylphosphatidyl serine, diacylphosphatidyl ethanolamine, diacylphosphatidyl glycerol, diacylphosphatidyl glycerol, etc.) as well as fluorinated derivatives of fatty acyl esters and free fatty acids may also function in accordance with the scope of the invention. Additionally lipid based and fluorinated (including perfluorinated) surfactants may be used as stabilizing materials in the present invention.
- 20 Examples of polymerized lipids include unsaturated lipophilic chains such as alkenyl or alkynyl, containing up to about 50 carbon atoms. Further examples are phospholipids such as phosphoglycerides and sphingolipids carrying polymerizable groups; and saturated and unsaturated fatty acid derivatives with hydroxyl groups, such as for example triglycerides of d-12-hydroxyoleic acid, including castor oil and ergot oil.
- 25 Polymerization may be designed to include hydrophilic substituents such as carboxyl or hydroxyl groups, to enhance dispersability so that the backbone residue resulting from

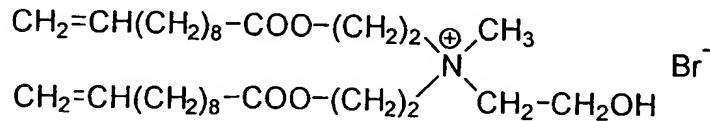
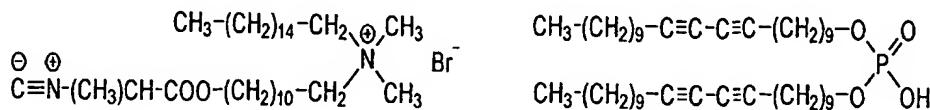
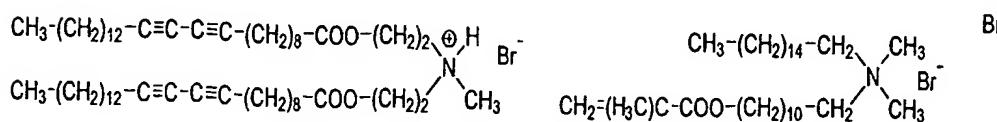
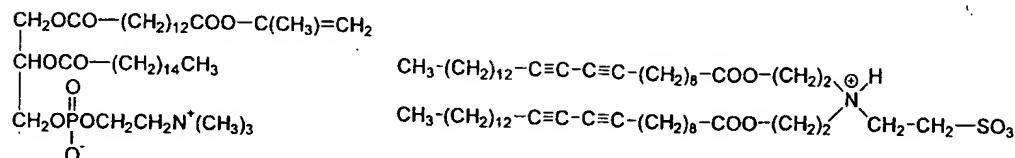
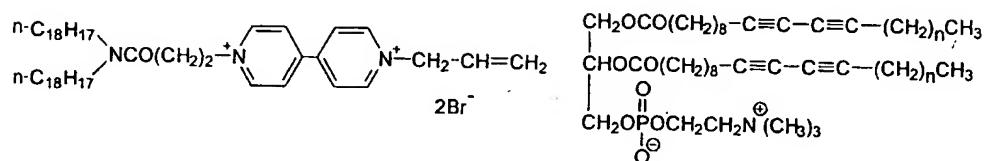
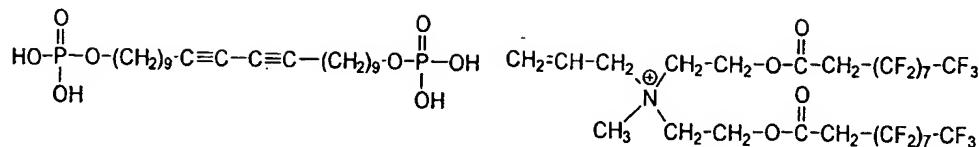
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biodegradation is water soluble. Suitable polymerizable lipids are also described, for example, by Klaveness et al, U.S. Patent No. 5,536,490, the disclosure of which is hereby incorporated by reference herein in its entirety.

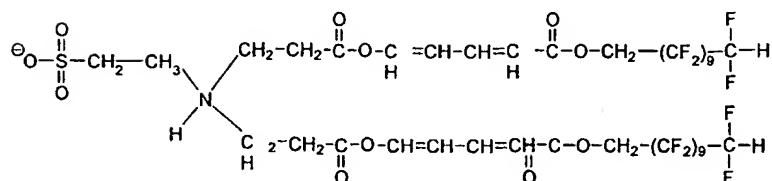
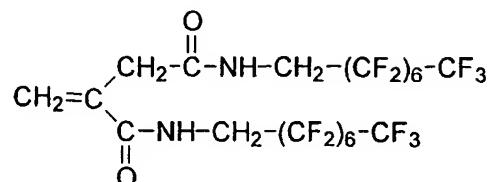
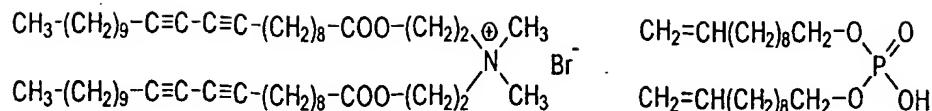
Exemplary polymerizable and/or fluorinated lipid compounds which may
5 be utilized in the compositions of the present invention are illustrated below.



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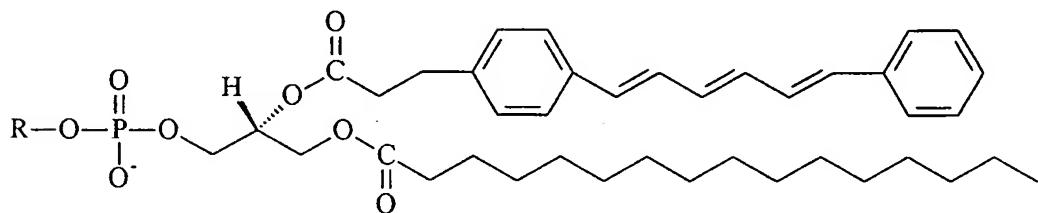


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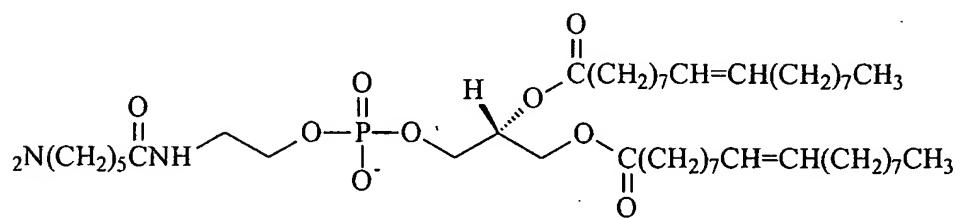
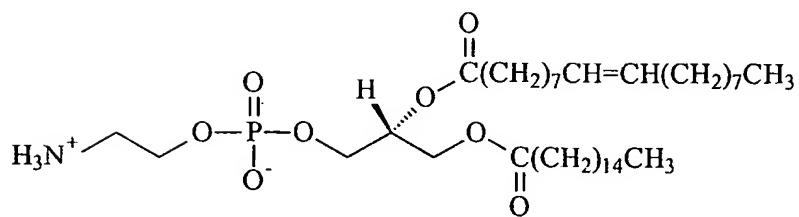
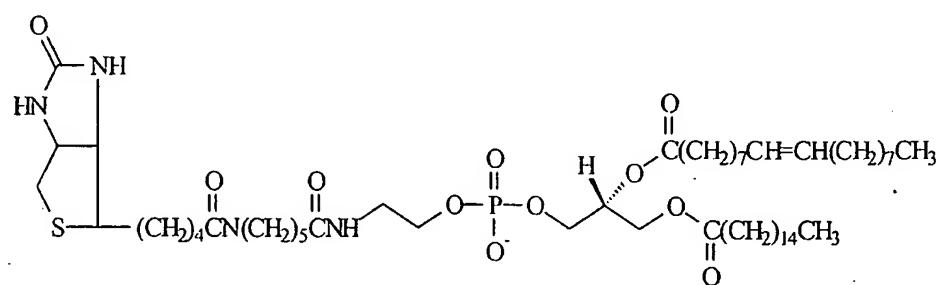
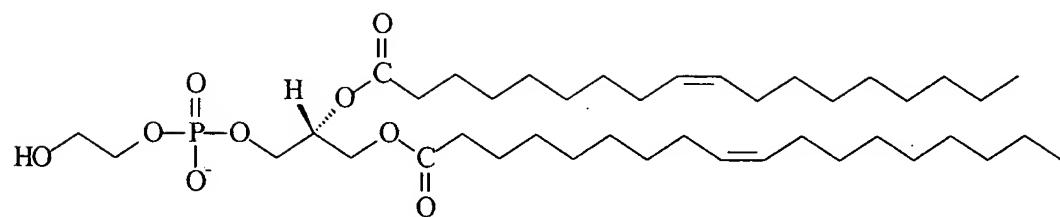
In formula A, above, x is an integer from about 8 to about 18, and n is $2x$. Most preferably x is 12 and n is 24. In formulas B, C, K and L, above, m, n, m' and n' are, independently, an integer of from about 8 to about 18, preferably about 10 to about 14.

5 Other lipids which may be employed in the present compositions include,
for example,

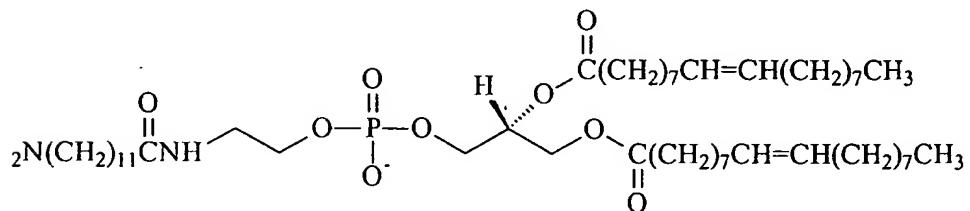


where R is choline,

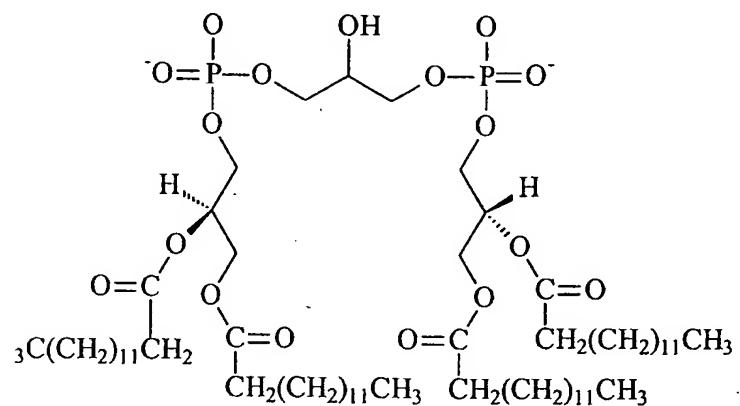
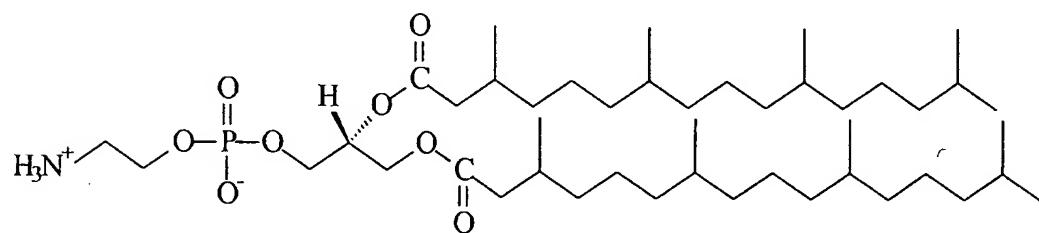
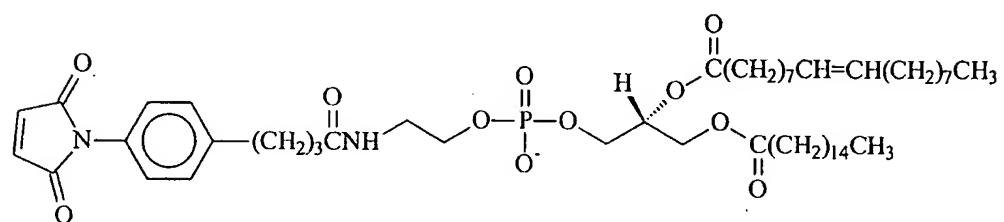
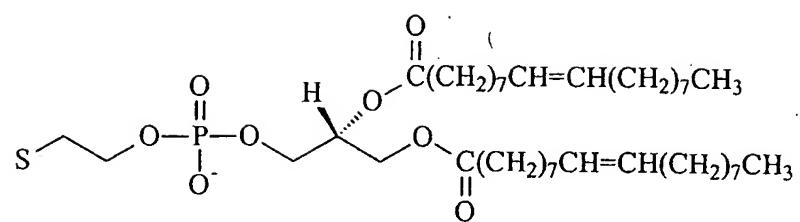
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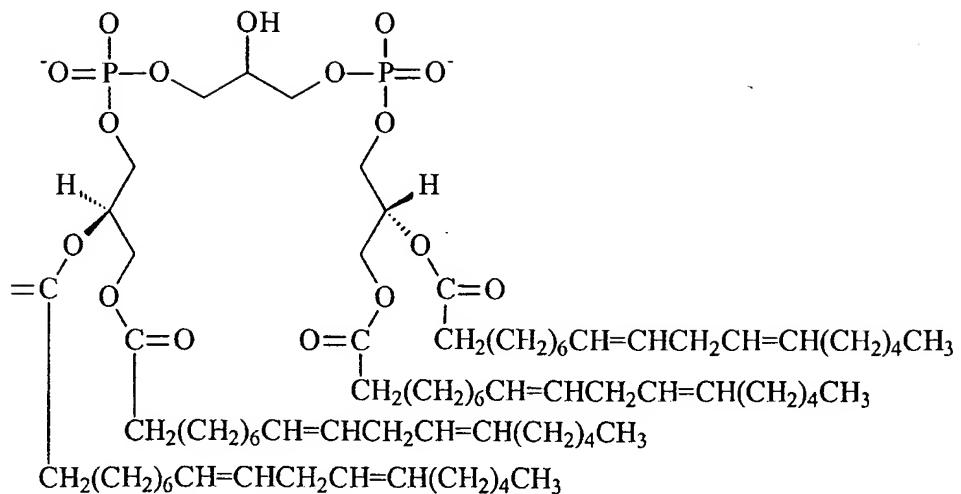
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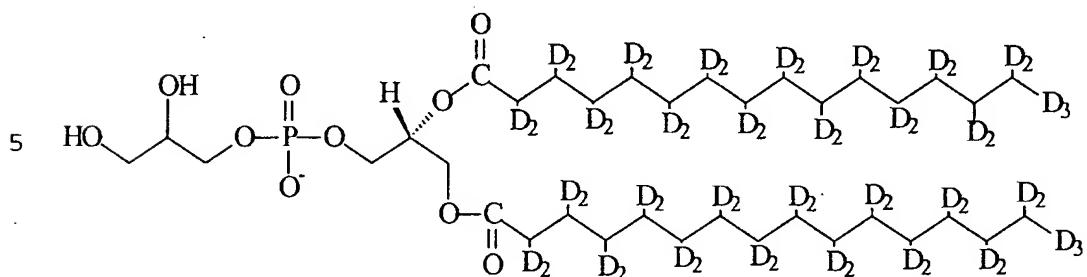
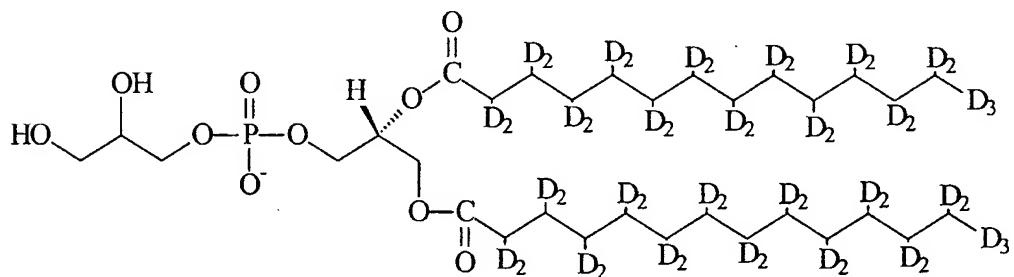
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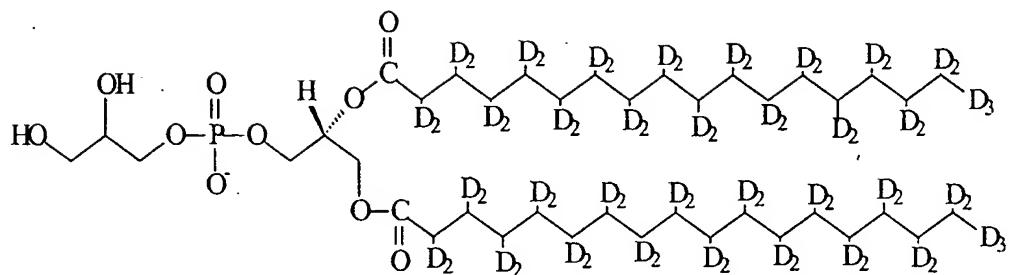
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deuterated lipids, such as



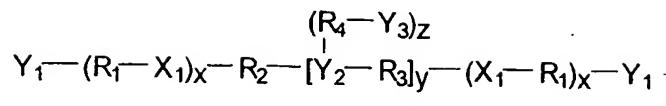
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If desired, a cationic lipid may be used, such as, for example, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleyloxy-3-(trimethylammonio)propane (DOTAP); and 1,2-dioleoyl-3-(4'-trimethylammonio)-
5 butanoyl-sn-glycerol (DOTB). If a cationic lipid is employed in the lipid compositions, the molar ratio of cationic lipid to non-cationic lipid may be, for example, from about 1:1000 to about 1:100. Preferably, the molar ratio of cationic lipid to non-cationic lipid may be from about 1:2 to about 1:10, with a ratio of from about 1:1 to about 1:2.5 being preferred. Even more preferably, the molar ratio of cationic lipid to non-cationic lipid may
10 be about 1:1.

In the case of lipid compositions which contain both cationic and non-cationic lipids, a wide variety of lipids may be employed as the non-cationic lipid. Preferably, this non-cationic lipid comprises one or more of DPPC, DPPE and dioleoylphosphatidylethanolamine. In lieu of the cationic lipids listed above, lipids
15 bearing cationic polymers, such as polylysine or polyarginine, as well as alkyl phosphonates, alkyl phosphinates, and alkyl phosphites, may also be used in the lipid compositions

In certain preferred embodiments of the present invention, the lipid compositions may comprise one or more cationic lipids having the following formula



(I)

wherein:

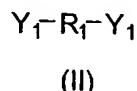
- each of x, y and z is independently an integer from 0 to about 100;
- each X_1 is independently $-\text{O}-$, $-\text{S}-$, $-\text{NR}_5-$, $-\text{C}(=\text{X}_2)-$, $-\text{C}(=\text{X}_2)-\text{N}(\text{R}_5)-$, $-\text{N}(\text{R}_5)-\text{C}(=\text{X}_2)-$, $-\text{C}(=\text{X}_2)-\text{O}-$, $-\text{O}-\text{C}(=\text{X}_2)-$ or $-\text{X}_2-(\text{R}_5\text{X}_2)\text{P}(=\text{X}_2)-\text{X}_2-$;
- 5 each X_2 is independently O or S;
- each Y_1 is independently a phosphate residue, $\text{N}(\text{R}_6)_a-$, $\text{S}(\text{R}_6)_a-$, $\text{P}(\text{R}_6)_a-$ or $-\text{CO}_2\text{R}_6$, wherein a is an integer from 1 to 3;
- each Y_2 is independently $-\text{N}(\text{R}_6)_b-$, $-\text{S}(\text{R}_6)_b-$ or $-\text{P}(\text{R}_6)_b-$, wherein b is an integer from 0 to 2;
- 10 each Y_3 is independently a phosphate residue, $\text{N}(\text{R}_6)_a-$, $\text{S}(\text{R}_6)_a-$, $\text{P}(\text{R}_6)_a-$ or $-\text{CO}_2\text{R}_6$, wherein a is an integer from 1 to 3;
- each of R_1 , R_2 , R_3 and R_4 is independently alkylene of 1 to about 20 carbons;
- 15 each R_5 is independently hydrogen or alkyl of 1 to about 10 carbons; and each R_6 is independently $-\text{[R}_7-\text{X}_3\text{]}_c-\text{R}_8$ or $-\text{R}_9-\text{[X}_4-\text{R}_{10}\text{]}_d-\text{Q}$, wherein:
 - each of c and d is independently an integer from 0 to about 100;
 - each Q is independently a phosphate residue, $-\text{N}(\text{R}_{11})_q-$, $-\text{S}(\text{R}_{11})_q-$, $-\text{P}(\text{R}_{11})_q-$ or $-\text{CO}_2\text{R}_6$, wherein q is an integer from 1 to 3;
 - each of X_3 and X_4 is independently $-\text{O}-$, $-\text{S}-$, $-\text{NR}_5-$, $-\text{C}(=\text{X}_2)-$, $-\text{C}(=\text{X}_2)-\text{N}(\text{R}_5)-$, $-\text{N}(\text{R}_5)-\text{C}(=\text{X}_2)-$, $-\text{C}(=\text{X}_2)-\text{O}-$, $-\text{O}-\text{C}(=\text{X}_2)-$ or $-\text{X}_2-(\text{R}_5\text{X}_2)\text{P}(=\text{X}_2)-\text{X}_2-$;
 - each R_7 is independently alkylene of 1 to about 20 carbons;
 - each R_8 is independently hydrogen or alkyl of 1 to about 60 carbons;
 - each of R_9 and R_{10} is independently alkylene of 1 to about 20 carbons; and each R_{11} is independently $-\text{[R}_7-\text{X}_3\text{]}_c-\text{R}_8$ or $-\text{R}_9-\text{[X}_4-\text{R}_{10}\text{]}_d-\text{W}$, wherein:

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each W is independently a phosphate residue, -N(R₁₂)_w, -S(R₁₂)_w, -P(R₁₂)_w or -CO₂R₆, wherein w is an integer from 1 to 3; and

R₁₂ is -[R₇-X₃]_c-R₈, with the proviso that the compound of formula (I) comprises at least one, and preferably at least two, quaternary salts.

- 5 Another cationic lipid compound which may be incorporated in the compositions of the present invention is a compound of the formula



wherein:

each Y₁ is independently a phosphate residue, N(R₂)_a-, S(R₂)_a-, P(R₂)_a- or -CO₂R₂, wherein a is an integer from 1 to 3;

- 10 R₁ is alkylene of 1 to about 60 carbons containing 0 to about 30 -O-, -S-, -NR₃- or -X₂-(R₃X₂)P(=X₂)-X₂- heteroatoms or heteroatom groups;

R₂ is a residue of the formula -R₄-[(X₁-R₅)_x-Y₂]_y-R₆, wherein:

each of x and y is independently an integer from 0 to about 100;

each X₁ is independently a direct bond, -O-, -S-, -NR₃-, -C(=X₂)-,

- 15 -C(=X₂)-N(R₃)-, -N(R₃)-C(=X₂)-, -C(=X₂)-O-, -O-C(=X₂)- or -X₂-(R₃X₂)P(=X₂)-X₂-;

each X₂ is independently O or S;

- each Y₂ is independently -S(R₂)_b-, -N(R₂)_b or -P(R₂)_b-, wherein b is an integer from 0 to 2;

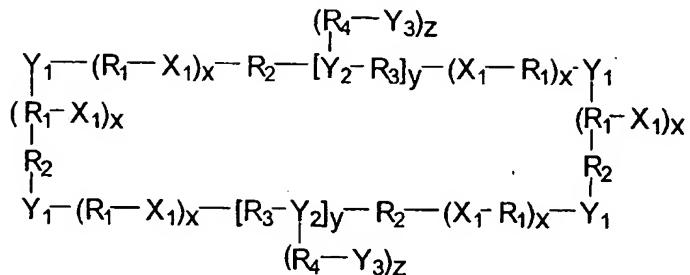
each R₃ is independently hydrogen or alkyl of 1 to about 10 carbons;

- 20 each of R₄ and R₅ is independently a direct bond or alkylene of 1 to about 30 carbons containing 0 to about 15 -O-, -S-, -NR₃- or -X₂-(R₃X₂)P(=X₂)-X₂- heteroatoms or heteroatom groups; and

- each R₆ is independently hydrogen or alkyl of 1 to about 60 carbons containing 0 to about 30 -O-, -S-, -NR₃- or -X₂-(R₃X₂)P(=X₂)-X₂- heteroatoms or heteroatom groups; with the proviso that the compound of formula (II) comprises at least one, and preferably at least two, quaternary salts.

- 40 -

In yet another embodiment, the present lipid compositions may comprise a cationic lipid compound of the formula



(III)

wherein:

- each of x, y and z is independently an integer from 0 to about 100;
- 5 each X_1 is independently -O-, -S-, -NR₅-, -C(=X₂)-, -C(=X₂)-N(R₅)-, -N(R₅)-C(=X₂)-, -C(=X₂)-O-, -O-C(=X₂)- or -X₂-(R₅X₂)P(=X₂)-X₂-;
- each X_2 is independently O or S;
- each Y_1 is independently -O-, -N(R₆)_a-, -S(R₆)_a- or -P(R₆)_a-, wherein a is an integer from 0 to 2;
- 10 each Y_2 is independently -N(R₆)_a-, -S(R₆)_a- or -P(R₆)_a-, wherein a is an integer from 0 to 2;
- each Y_3 is independently a phosphate residue, N(R₆)_b-, S(R₆)_b-, P(R₆)_b- or -CO₂R₆, wherein b is an integer from 1 to 3;
- each of R₁, R₂, R₃ and R₄ is independently alkylene of 1 to about 20 carbons;
- 15 each R₅ is independently hydrogen or alkyl of 1 to about 10 carbons; and each R₆ is independently -[R₇-X₃]_c-R₈ or -R₉-[X₄-R₁₀]_d-Q, wherein:
 - each of c and d is independently an integer from 0 to about 100;
 - each Q is independently a phosphate residue, -N(R₁₁)_q-, -S(R₁₁)_q-, -P(R₁₁)_q or -CO₂R₁₁, wherein q is an integer from 1 to 3;
 - each of X₃ and X₄ is independently -O-, -S-, -NR₅-, -C(=X₂)-, -C(=X₂)-N(R₅)-, -N(R₅)-C(=X₂)-, -C(=X₂)-O-, -O-C(=X₂)- or -X₂-(R₅X₂)P(=X₂)-X₂-;

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- each R₇ is independently alkylene of 1 to about 20 carbons;
each R₈ is independently hydrogen or alkyl of 1 to about 60 carbons;
each of R₉ and R₁₀ is independently alkylene of 1 to about 20 carbons; and
each R₁₁ is independently -[R₇-X₃]_c-R₈ or -R₉-[X₄-R₁₀]_d-W, wherein:
5 each W is independently a phosphate residue, -N(R₁₂)_w, -S(R₁₂)_w, -P(R₁₂)_w
or -CO₂R₁₂, wherein w is an integer from 1 to 3; and

- R₁₂ is -[R₇-X₃]_c-R₈; with the proviso that the compound of formula (III)
comprises at least one, and preferably at least two, quaternary salts. The cationic lipid
compounds which are described generically above are set forth in U.S. Patent No.
10 5,830,430, the disclosures of which are hereby incorporated by reference herein, in their
entirety.

In certain preferred embodiments, the lipid compositions comprise
phospholipids, particularly one or more of DPPC, DPPE, DPPA, DSPC, DSPE, DSPG,
and DAPC (20 carbons).

- 15 In addition, saturated and unsaturated fatty acids may be employed in the
present lipid compositions may include molecules that preferably contain from about 12
carbons to about 22 carbons, in linear or branched form. Hydrocarbon groups consisting
of isoprenoid units and/or prenyl groups can be used as well. Examples of saturated fatty
acids that are suitable include, for example, lauric, myristic, palmitic, and stearic acids.
20 Suitable unsaturated fatty acids that may be used include, for example, lauroleic,
physeteric, myristoleic, palmitoleic, petroselinic, and oleic acids. Examples of branched
fatty acids that may be used include, for example, isolauric, isomyristic, isopalmitic, and
isostearic acids. Other lipids which may be employed in the present compositions include
those disclosed in U.S. application Serial No. 08/851,780, filed May 6, 1997 and U.S.
25 application Serial No. 08/887,215, filed July 2, 1997, the disclosures of which are hereby
incorporated herein by reference, in their entireties.

- In addition to lipid compositions and/or vesicle compositions formulated
from lipids, embodiments of the present invention may also involve vesicles formulated
from proteins or derivatives thereof. Vesicles which are formulated from proteins that
30 may be used to prepare the targeted vesicles of the present invention are described, for
example, in Feinstein, U.S. Patent Nos. 4,572,203, 4,718,433 and 4,774,958, and Cerny et

al., U.S. Patent No. 4,957,656. Other protein-based vesicles, in addition to those described in the aforementioned patents, would be apparent to one of ordinary skill in the art, once armed with the present disclosure.

- In addition to lipid compositions and/or vesicle compositions formulated from lipids and/or proteins, embodiments of the present invention may also involve vesicles formulated from polymers which may be of natural, semi-synthetic (modified natural) or synthetic origin. As used herein, the term polymer denotes a compound comprised of two or more repeating monomeric units, and preferably 10 or more repeating monomeric units. The phrase semi-synthetic polymer (or modified natural polymer), as employed herein, denotes a natural polymer that has been chemically modified in some fashion. Exemplary natural polymers suitable for use in the present invention include naturally occurring polysaccharides. Such polysaccharides include, for example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galatocarolose, pectic acid, pectins, including amylose, pullulan, glycogen, amylopectin, cellulose, dextran, dextrin, dextrose, polydextrose, pustulan, chitin, agarose, keratan, chondroitan, dermatan, hyaluronic acid, alginic acid, xanthan gum, starch and various other natural homopolymer or heteropolymers, such as those containing one or more of the following aldoses, ketoses, acids or amines: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, mannitol, sorbitol, lactose, sucrose, trehalose, maltose, cellobiose, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine, and neuraminic acid, and naturally occurring derivatives thereof. Accordingly, suitable polymers include, for example, proteins, such as albumin. Exemplary semi-synthetic polymers include carboxymethylcellulose, hydroxymethylcellulose, hydroxypropylmethylcellulose, methylcellulose, and methoxycellulose. Exemplary synthetic polymers suitable for use in the present invention include polyethylenes (such as, for example, polyethylene glycol, polyoxyethylene, and polyethylene terephthalate), polypropylenes (such as, for example, polypropylene glycol), polyurethanes (such as, for example, polyvinyl alcohol (PVA),

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- polyvinyl chloride and polyvinylpyrrolidone), polyamides including nylon, polystyrene, polylactic acids, fluorinated hydrocarbons, fluorinated carbons (such as, for example, polytetrafluoroethylene), and polymethylmethacrylate, and derivatives thereof. Preferred are biocompatible synthetic polymers or copolymers prepared from monomers, such as
- 5 acrylic acid, methacrylic acid, ethyleneimine, crotonic acid, acrylamide, ethyl acrylate, methyl methacrylate, 2-hydroxyethyl methacrylate (HEMA), lactic acid, glycolic acid, ϵ -caprolactone, acrolein, cyanoacrylate, bisphenol A, epichlorhydrin, hydroxyalkyl-acrylates, siloxane, dimethylsiloxane, ethylene oxide, ethylene glycol, hydroxyalkyl-methacrylates, N-substituted acrylamides, N-substituted methacrylamides, N-vinyl-2-
- 10 pyrrolidone, 2,4-pentadiene-1-ol, vinyl acetate, acrylonitrile, styrene, p-amino-styrene, p-amino-benzyl-styrene, sodium styrene sulfonate, sodium 2-sulfoxyethylmethacrylate, vinyl pyridine, aminoethyl methacrylates, 2-methacryloyloxy-trimethylammonium chloride, and polyvinylidene, as well polyfunctional crosslinking monomers such as N,N'-methylenebisacrylamide, ethylene glycol dimethacrylates, 2,2'-(p-phenylenedioxy)-diethyl
- 15 dimethacrylate, divinylbenzene, triallylamine and methylenebis-(4-phenyl-isocyanate), including combinations thereof. Preferable polymers include polyacrylic acid, polyethyleneimine, polymethacrylic acid, polymethylmethacrylate, polysiloxane, polydimethylsiloxane, polylactic acid, poly(ϵ -caprolactone), epoxy resin, poly(ethylene oxide), poly(ethylene glycol), and polyamide (nylon) polymers. Preferable copolymers
- 20 include the following: polyvinylidene-polyacrylonitrile, polyvinylidene-polyacrylonitrile-polymethylmethacrylate, polystyrene-polyacrylonitrile and poly d-1, lactide co-glycolide polymers. A preferred copolymer is polyvinylidene- polyacrylonitrile. Other suitable biocompatible monomers and polymers will be readily apparent to those skilled in the art, once armed with the present disclosure.
- 25 As noted above, the present lipid compositions also preferably comprise a gas, such as an inert gas. The gas provides the lipid compositions with enhanced reflectivity, particularly in connection with vesicle compositions in which the gas is entrapped within the vesicles. This may increase their effectiveness as contrast agents. Preferred gases are gases which are inert and which are biocompatible, that
- 30 is, gases which are not injurious to biological function. Preferred gases include those selected from the group consisting of air, noble gases, such as helium, rubidium

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hyperpolarized xenon, hyperpolarized argon, hyperpolarized helium, neon, argon and xenon, carbon dioxide, nitrogen, fluorine, oxygen, sulfur-based gases, such as sulfur hexafluoride and sulfur tetrafluoride, fluorinated gases, including, for example, partially fluorinated gases or completely fluorinated gases. Exemplary fluorinated gases include the

5 fluorocarbon gases, such as the perfluorocarbon gases, and mixtures thereof. Paramagnetic gases, such as $^{17}\text{O}_2$ may also be used in the lipid compositions.

In preferred embodiments, the gas comprises a fluorinated gas. Such fluorinated gases include materials which contain one, or more than one, fluorine atom. Preferred are gases which contain more than one fluorine atom, with perfluorocarbons

10 (that is, fully fluorinated fluorocarbons) being more preferred. Preferably, the perfluorocarbon gas is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, perfluorocyclobutane and mixtures thereof. More preferably, the perfluorocarbon gas is perfluoropropane or perfluorobutane, with perfluorobutane being particularly preferred.

15 Another preferable gas is sulfur hexafluoride. Yet another preferable gas is heptafluoropropane, including 1,1,1,2,3,3-heptafluoropropane and its isomer, 1,1,2,2,3,3-heptafluoropropane. It is contemplated that mixtures of different types of gases, such as mixtures of a perfluorocarbon gas and another type of gas, such as air, can also be used in the compositions of the present invention. Other gases, including the gases

20 exemplified above, would be readily apparent to one skilled in the art based on the present disclosure.

In certain preferred embodiments, a gas, for example, air or a perfluorocarbon gas, is combined with a liquid perfluorocarbon, such as perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane, perfluorononane,

25 perfluorooctylbromide (PFOB), perfluorodecalin, perfluorododecalin, perfluoroctyliodide, perfluorotripropylamine and perfluorotributylamine.

It may also be desirable to incorporate in the lipid compositions a precursor to a gaseous substance. Such precursors include materials that are capable of being converted to a gas *in vivo*. Preferably, the gaseous precursor is biocompatible, and the gas

30 produced *in vivo* is biocompatible also.

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Among the gaseous precursors which are suitable for use in compositions described herein are agents which are sensitive to pH. These agents include materials that are capable of evolving gas, for example, upon being exposed to a pH that is neutral or acidic. Examples of such pH sensitive agents include salts of an acid which is selected 5 from the group consisting of inorganic acids, organic acids and mixtures thereof. Carbonic acid (H_2CO_3) is an example of a suitable inorganic acid, and aminomalonic acid is an example of a suitable organic acid. Other acids, including inorganic and organic acids, would be readily apparent to one skilled in the art based on the present disclosure.

Gaseous precursors which are derived from salts are preferably selected 10 from the group consisting of alkali metal salts, ammonium salts and mixtures thereof. More preferably, the salt is selected from the group consisting of carbonate, bicarbonate, sesquicarbonate, aminomalonate and mixtures thereof.

Examples of suitable gaseous precursor materials which are derived from salts include, for example, lithium carbonate, sodium carbonate, potassium carbonate, 15 lithium bicarbonate, sodium bicarbonate, potassium bicarbonate, magnesium carbonate, calcium carbonate, magnesium bicarbonate, ammonium carbonate, ammonium bicarbonate, ammonium sesquicarbonate, sodium sesquicarbonate, sodium aminomalonate and ammonium aminomalonate. Aminomalonate is well known in the art, and its preparation is described, for example, in Thanassi, *Biochemistry*, Vol. 9, no. 3, pp. 20 525-532 (1970); Fitzpatrick et al., *Inorganic Chemistry*, Vol. 13, no. 3 pp. 568-574 (1974); and Stelmashok et al., *Koordinatsionnaya Khimiya*, Vol. 3, no. 4, pp. 524-527 (1977). The disclosures of these publications are hereby incorporated herein by reference.

In addition to, or instead of, being sensitive to changes in pH, the gaseous precursor materials may also comprise compounds which are sensitive to changes in 25 temperature. Exemplary of suitable gaseous precursors which are sensitive to changes in temperature are the perfluorocarbons. As the artisan will appreciate, a particular perfluorocarbon may exist in the liquid state when the lipid compositions are first made, and are thus used as a gaseous precursor. Alternatively, the perfluorocarbon may exist in the gaseous state when the lipid compositions are made, and are thus used directly as a gas. 30 Whether the perfluorocarbon is used as a liquid or a gas generally depends on its liquid/gas phase transition temperature, or boiling point. For example, a preferred perfluorocarbon,

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- perfluoropentane, has a liquid/gas phase transition temperature (boiling point) of 29.5°C. This means that perfluoropentane is generally a liquid at room temperature (about 25°C), but is converted to a gas within the human body, the normal temperature of which is about 37°C, which is above the transition temperature of perfluoropentane. Thus, under normal
- 5 circumstances, perfluoropentane is a gaseous precursor. As a further example, there are the homologs of perfluoropentane, namely perfluorobutane and perfluorohexane. The liquid/gas transition of perfluorobutane is 4°C and that of perfluorohexane is 57°C. Thus, perfluorobutane can be useful as a gaseous precursor, although more likely as a gas, whereas perfluorohexane can be useful as a gaseous precursor because of its relatively
- 10 high boiling point. As known to one of ordinary skill in the art, the effective boiling point of a substance may be related to the pressure to which that substance is exposed. This relationship is exemplified by the ideal gas law: $PV = nRT$, where P is pressure, V is volume, n is moles of substance, R is the gas constant, and T is temperature. The ideal gas law indicates that as pressure increases, the effective boiling point increases also.
- 15 Conversely, as pressure decreases, the effective boiling point decreases.

A wide variety of materials can be used as gaseous precursors in the present compositions. It is only required that the material be capable of undergoing a phase transition to the gas phase upon passing through the appropriate temperature. Suitable gaseous precursors include, for example, hexafluoroacetone, isopropyl acetylene, allene,

20 tetrafluoroallene, boron trifluoride, 1,2-butadiene, 2,3-butadiene, 1,3-butadiene, 1,2,3-trichloro-2-fluoro-1,3-butadiene, 2-methyl-1,3-butadiene, hexafluoro-1,3-butadiene, butadiyne, 1-fluorobutane, 2-methylbutane, perfluorobutane, 1-butene, 2-butene, 2-methyl-1-butene, 3-methyl-1-butene, perfluoro-1-butene, perfluoro-2-butene, 4-phenyl-3-butene-2-one, 2-methyl-1-butene-3-yne, butyl nitrate, 1-butyne, 2-butyne, 2-chloro-1,1,1,4,4,4-

25 hexafluorobutyne, 3-methyl-1-butyne, perfluoro-2-butyne, 2-bromo-butyraldehyde, carbonyl sulfide, crotononitrile, cyclobutane, methylcyclobutane, octafluorocyclobutane, perfluorocyclobutene, 3-chlorocyclopentene, perfluorocyclo-pentane, octafluorocyclo-pentene, cyclopropane, perfluorocyclopropane, 1,2-dimethyl-cyclopropane, 1,1-dimethyl-cyclopropane, 1,2-dimethylcyclopropane, ethylcyclopropane, methylcyclopropane,

30 diacetylene, 3-ethyl-3-methyl diaziridine, 1,1,1-trifluorodiazooethane, dimethyl amine, hexafluorodimethylamine, dimethylethylamine, bis(dimethylphosphine)-amine,

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- perfluorohexane, perfluoroheptane, perfluoroctane, 2,3-dimethyl-2-norbornane,
perfluorodimethylamine, dimethyloxonium chloride, 1,3-dioxolane-2-one, 4-methyl-
1,1,1,2-tetrafluoroethane, 1,1,1-trifluoroethane, 1,1,2,2-tetrafluoroethane, 1,1,2-trichloro-
1,2,2-trifluoroethane, 1,1-dichloroethane, 1,1-dichloro-1,2,2,2-tetrafluoroethane,
5 1,2-difluoroethane, 1-chloro-1,1,2,2,2-pentafluoroethane, 2-chloro-1,1-difluoroethane,
1,1-dichloro-2-fluoroethane, 1-chloro-1,1,2,2-tetrafluoroethane, 2-chloro-1,1-
difluoroethane, chloroethane, chloropentafluoroethane, dichlorotrifluoroethane,
fluoroethane, perfluoroethane, nitropentafluoroethane, nitrosopentafluoroethane,
perfluoroethylamine, ethyl vinyl ether, 1,1-dichloroethane, 1,1-dichloro-1,2-
10 difluoroethane, 1,2-difluoroethane, methane, trifluoromethanesulfonylchloride,
trifluoromethanesulfonylfluoride, bromodifluoronitrosomethane, bromofluoromethane,
bromochlorofluoromethane, bromotrifluoromethane, chlorodifluoronitromethane,
chlorodinitromethane, chlorofluoromethane, chlorotrifluoromethane,
chlorodifluoromethane, dibromodifluoromethane, dichlorodifluoromethane,
15 dichlorofluoromethane, difluoromethane, difluoroiodomethane, disilanomethane,
fluoromethane, iodomethane, iodotrifluoromethane, nitrotrifluoromethane,
nitrosotrifluoromethane, tetrafluoromethane, trichlorofluoromethane, trifluoromethane,
2-methylbutane, methyl ether, methyl isopropyl ether, methyl lactate, methyl nitrite,
methyl sulfide, methyl vinyl ether, neopentane, nitrous oxide, 1,2,3-nonadecane-
20 tricarboxylic acid 2-hydroxytrimethyl ester, 1-nonene-3-ync, 1,4-pentadiene, *n*-pentane,
perfluoropentane, 4-amino-4-methylpentan-2-one, 1-pentene, 2-pentene (cis and trans),
3-bromopent-1-ene, perfluoropent-1-ene, tetrachlorophthalic acid, 2,3,6-trimethyl-
piperidine, propane, 1,1,1,2,2,3-hexafluoropropane, 1,2-epoxypropane, 2,2-difluoro-
propane, 2-aminopropane, 2-chloropropane, heptafluoro-1-nitropropane, heptafluoro-1-
25 nitrosopropane, perfluoropropane, propene, hexafluoropropane, 1,1,1,2,3,3-hexafluoro-2,3-
dichloropropane, 1-chloropropane, chloropropane-(trans), 2-chloropropane,
3-fluoropropane, propyne, 3,3,3-trifluoropropyne, 3-fluorostyrene, sulfur (di)-decafluoride
(S₂F₁₀), 2,4-diaminotoluene, trifluoroacetonitrile, trifluoromethyl peroxide, trifluoromethyl
sulfide, tungsten hexafluoride, vinyl acetylene and vinyl ether.
- 30 Perfluorocarbons are both preferred gases and preferred gaseous precursors
for use in connection with the compositions employed in the methods of the present

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invention. Included among such perfluorocarbons are saturated perfluorocarbons, unsaturated perfluorocarbons, and cyclic perfluorocarbons. The saturated perfluorocarbons, which are usually preferred, have the formula C_nF_{2n+2} , where n is from 1 to about 12, preferably about 2 to about 10, more preferably about 3 to about 8, and even 5 more preferably about 3 to about 6. Suitable perfluorocarbons include, for example, perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluorocyclobutane, perfluoropentane, perfluorohexane, perfluoroheptane, perfluorooctane and perfluorononane. Preferably, the perfluorocarbon is selected from the group consisting of perfluoropropane, perfluorobutane, perfluorocyclobutane, 10 perfluoropentane, perfluorohexane and perfluorooctane, with perfluorobutane being particularly preferred. Cyclic perfluorocarbons, which have the formula C_nF_{2n} , where n is from 3 to 8, preferably 3 to 6, may also be preferred, and include, for example, hexafluorocyclopropane, octafluorocyclobutane, and decafluorocyclopentane.

In addition to the perfluorocarbons, it may be desirable to utilize stable 15 fluorocarbons which are not completely fluorinated. Such fluorocarbons include heptafluoropropane, for example, 1,1,1,2,3,3,3-heptafluoropropane and its isomer, 1,1,2,2,3,3,3-heptafluoropropane.

The gaseous precursor materials may be also photoactivated materials, such 20 as diazonium ion and aminomalonate. As discussed more fully hereinafter, certain lipid and/or vesicle compositions, and particularly vesicle compositions, may be formulated so that gas is formed at the target tissue or by the action of sound on the lipid composition. Examples of gaseous precursors are described, for example, in U.S. Patent Nos. 5,088,499 and 5,149,319, the disclosures of which are hereby incorporated herein by reference, in their entirety. Other gaseous precursors, in addition to those exemplified above, will be 25 apparent to one skilled in the art based on the present disclosure.

The gaseous substances and/or gaseous precursors are preferably incorporated in the lipid and/or vesicle compositions irrespective of the physical nature of the composition. Thus, it is contemplated that the gaseous substances and/or precursors thereto may be incorporated, for example, in lipid compositions in which the lipids are 30 aggregated randomly, as well as in vesicle compositions, including vesicle compositions which are formulated from lipids, such as micelles and liposomes. Incorporation of the

gaseous substances and/or precursors thereto in the lipid and/or vesicle compositions may be achieved by using any of a number of methods. For example, in the case of vesicles based on lipids, the formation of gas filled vesicles can be achieved by shaking or otherwise agitating an aqueous mixture which comprises a gas or gaseous precursor and 5 one or more lipids. This promotes the formation of stabilized vesicles within which the gas or gas precursor is encapsulated.

In addition, a gas may be bubbled directly into an aqueous mixture of lipid and/or vesicle-forming compounds. Alternatively, a gas instillation method can be used as disclosed, for example, in U.S. Patent Nos. 5,352,435 and 5,228,446, the disclosures of 10 which are hereby incorporated herein by reference, in their entirety. Suitable methods for incorporating the gas or gas precursor in cationic lipid compositions are disclosed also in U.S. Patent No. 4,865,836, the disclosures of which are hereby incorporated herein by reference. Other methods would be apparent to one skilled in the art based on the present disclosure. Preferably, the gas may be instilled in the lipid and/or vesicle compositions 15 after or during the addition of the stabilizing material and/or during formation of vesicles.

In preferred embodiments, the gaseous substances and/or gaseous precursor materials are incorporated in vesicle compositions, with micelles and liposomes being preferred. As discussed in detail below, vesicles in which a gas or gaseous precursor or both are encapsulated are advantageous in that they provide improved reflectivity *in vivo*.

20 As discussed more fully hereinafter, it is preferred that the lipid compositions, and especially the vesicle compositions, be formulated from lipids and optional stabilizing compounds to promote the formation of stable vesicles. In addition, it is also preferred that the lipid and/or vesicle compositions comprise a highly stable gas as well. The phrase "highly stable gas" refers to a gas which has limited solubility and 25 diffusability in aqueous media. Exemplary highly stable gases include perfluorocarbons since they are generally less diffusible and relatively insoluble in aqueous media. Accordingly, their use may promote the formation of highly stable vesicles.

In certain embodiments, it may be desirable to use a fluorinated compound, especially a perfluorocarbon compound, which may be in the liquid state at the 30 temperature of use of the lipid and/or vesicle compositions, including, for example, the *in vivo* temperature of the human body, to assist or enhance the stability of the lipid and/or

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vesicle compositions, and especially, the gas filled vesicles. Suitable fluorinated compounds include, for example, fluorinated surfactants, such as fluorinated surfactants which are commercially available as ZONYL® surfactants (the DuPont Company, Wilmington, DE), as well as liquid perfluorocarbons, such as for example,

5 perfluoroctylbromide (PFOB), perfluorodecalin, perfluorododecalin, perfluoroctyliodide, perfluorotripropylamine, and perfluorotributylamine. In general, perfluorocarbons comprising about six or more carbon atoms will be liquids at normal human body temperature. Among these perfluorocarbons, perfluoroctylbromide and perfluorohexane, which are liquids at room temperature, are preferred. The gas which is

10 present may be, for example, nitrogen or perfluoropropane, or may be derived from a gaseous precursor, which may also be a perfluorocarbon, for example, perfluoropentane. In the latter case, the lipid and/or vesicle compositions may be prepared from a mixture of perfluorocarbons, which for the examples given, would be perfluoropropane (gas) or perfluoropentane (gaseous precursor) and perfluoroctylbromide (liquid). Although not

15 intending to be bound by any theory or theories of operation, it is believed that, in the case of vesicle compositions, the liquid fluorinated compound may be situated at the interface between the gas and the membrane or wall surface of the vesicle. There may be thus formed a further stabilizing layer of liquid fluorinated compound on the internal surface of the stabilizing compound, for example, a biocompatible lipid used to form the vesicle, and

20 this perfluorocarbon layer may also prevent the gas from diffusing through the vesicle membrane. A gaseous precursor, within the context of the present invention, is a liquid at the temperature of manufacture and/or storage, but becomes a gas at least at or during the time of use.

Thus, it has been discovered that a liquid fluorinated compound, such as a

25 perfluorocarbon, when combined with a gas or gaseous precursor ordinarily used to make the lipid and/or vesicle compositions described herein, may confer an added degree of stability not otherwise obtainable with the gas or gaseous precursor alone. Thus, it is within the scope of the present invention to utilize a gas or gaseous precursor, such as a perfluorocarbon gaseous precursor, for example, perfluoropentane, together with a

30 perfluorocarbon which remains liquid after administration to a patient, that is, whose liquid to gas phase transition temperature is above the body temperature of the patient, for

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example, perfluoroctylbromide. Perfluorinated surfactants, such as ZONYL® fluorinated surfactants, may be used to stabilize the lipid and/or vesicle compositions, and to act, for example, as a coating for vesicles. Preferred perfluorinated surfactants are the partially fluorinated phosphocholine surfactants. In these preferred fluorinated surfactants, the dual 5 alkyl compounds may be fluorinated at the terminal alkyl chains and the proximal carbons may be hydrogenated. These fluorinated phosphocholine surfactants may be used for making the targeted lipid and/or vesicle compositions of the present invention.

In connection with embodiments involving vesicle compositions, the size of the vesicles can be adjusted for the particular intended end use including, for example, 10 diagnostic and/or therapeutic use. The size of the vesicles may preferably range from about 30 nanometers (nm) to about 100 micrometers (μm) in diameter, and all combinations and subcombinations of ranges therein. More preferably, the vesicles have diameters of from about 100 nm to about 10 μm , with diameters of from about 200 nm to about 7 μm being even more preferred. In connection with particular uses, for example, 15 intravascular use, including magnetic resonance imaging of the vasculature, it may be preferred that the vesicles be no larger than about 30 μm in diameter, with smaller vesicles being preferred, for example, vesicles of no larger than about 12 μm in diameter. In certain preferred embodiments, the diameter of the vesicles may be about 7 μm or less, with vesicles having a mean diameter of about 5 μm or less being more preferred, and 20 vesicles having a mean diameter of about 3 μm or less being even more preferred. It is contemplated that these smaller vesicles may perfuse small vascular channels, such as the microvasculature, while at the same time providing enough space or room within the vascular channel to permit red blood cells to slide past the vesicles.

The size of the gas filled vesicles can be adjusted, if desired, by a variety of 25 procedures including, for example, shaking, microemulsification, vortexing, extrusion, filtration, sonication, homogenization, repeated freezing and thawing cycles, extrusion under pressure through pores of defined size, and similar methods.

As noted above, compositions employed herein may also include, with respect to their preparation, formation and use, gaseous precursors that can be activated to 30 change from a liquid or solid state into a gas by temperature, pH, light, and energy (such as ultrasound). The gaseous precursors may be made into gas by storing the precursors at

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reduced pressure. For example, a vial stored under reduced pressure may create a headspace of perfluoropentane or perfluorohexane gas, useful for creating a preformed gas prior to injection. Preferably, the gaseous precursors may be activated by temperature. Set forth below is a table listing a series of gaseous precursors which undergo phase transitions from liquid to gaseous states at relatively close to normal body temperature (37°C) or below, and the size of the emulsified droplets that would be required to form a vesicle of a maximum size of 10 µm.

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**Physical Characteristics of Gaseous Precursors and
Diameter of Emulsified Droplet to Form a 10 µm Vesicle***

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Compound	Molecular Weight	Boiling Point (° C)	Density	Diameter (µm) of emulsified droplet to make 10 micron vesicle
perfluoro pentane	288.04	28.5	1.7326	2.9
1-fluorobutane	76.11	32.5	0.67789	1.2
2-methyl butane (isopentane)	72.15	27.8	0.6201	2.6
2-methyl 1-butene	70.13	31.2	0.6504	2.9
2-methyl-2-butene	70.13	38.6	0.6623	2.5
1-butene-3-yne-2-methyl	66.10	34.0	0.6801	2.4
3-methyl-1-butyne	68.12	29.5	0.6660	2.5
octafluoro cyclobutane	200.04	-5.8	1.48	2.8
decafluoro butane	238.04	-2	1.517	3.0

TABLE 2
Physical Characteristics of Gaseous Precursors and
Diameter of Emulsified Droplet to Form a 10 µm Vesicle*

Compound	Molecular Weight	Boiling Point (° C)	Density	Diameter (µm) of emulsified droplet to make 10 micron vesicle
hexafluoro ethane	138.01	-78.1	1.607	2.7

*Source: Chemical Rubber Company Handbook of Chemistry and Physics, Robert C. Weast and David R. Lide, eds., CRC Press, Inc. Boca Raton, Florida (1989-1990).

5 The perfluorocarbons, as already indicated, are preferred for use as the gas or gaseous precursors, as well as additional stabilizing components.

As noted above, it is preferred to optimize the utility of the lipid and/or vesicle compositions, especially vesicle compositions formulated from lipids, by using gases of limited solubility. The phrase "limited solubility" refers to the ability of the gas to
10 diffuse out of the vesicles by virtue of its solubility in the surrounding aqueous medium. A greater solubility in the aqueous medium imposes a gradient with the gas in the vesicle such that the gas may have a tendency to diffuse out of the vesicle. A lesser solubility in the aqueous milieu, may, on the other hand, decrease or eliminate the gradient between the vesicle and the interface such that diffusion of the gas out of the vesicle may be impeded.
15 Preferably, the gas entrapped in the vesicle has a solubility less than that of oxygen, that is, about 1 part gas in about 32 parts water. See *Matheson Gas Data Book*, 1966, Matheson Company Inc. More preferably, the gas entrapped in the vesicle possesses a solubility in water less than that of air; and even more preferably, the gas entrapped in the vesicle possesses a solubility in water less than that of nitrogen.

20 It may be desirable, in certain embodiments, to formulate vesicles from substantially impermeable polymeric materials. In these embodiments, it is generally unnecessary to employ a gas which is highly insoluble also. For example, stable vesicle compositions which comprise substantially impermeable polymeric materials may be formulated with gases having higher solubilities, for example, air or nitrogen.

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In addition to, or instead of, the lipid, proteinaceous and/or polymeric compounds discussed above, the compositions described herein may comprise one or more stabilizing materials. Exemplary of such stabilizing materials are, for example, biocompatible polymers. The stabilizing materials may be employed to desirably assist in 5 the formation of vesicles and/or to assure substantial encapsulation of the gases or gaseous precursors. Even for relatively insoluble, non-diffusible gases, such as perfluoropropane or sulfur hexafluoride, improved vesicle compositions may be obtained when one or more stabilizing materials are utilized in the formation of the gas and gaseous precursor filled vesicles. These compounds may help improve the stability and the integrity of the vesicles 10 with regard to their size, shape and/or other attributes.

The terms "stable" or "stabilized", as used herein, means that the vesicles may be substantially resistant to degradation, including, for example, loss of vesicle structure or encapsulated gas or gaseous precursor, for a useful period of time. Typically, the vesicles employed in the present invention have a desirable shelf life, often retaining at 15 least about 90 % by volume of its original structure for a period of at least about two to three weeks under normal ambient conditions. In preferred form, the vesicles are desirably stable for a period of time of at least about 1 month, more preferably at least about 2 months, even more preferably at least about 6 months, still more preferably about eighteen months, and yet more preferably up to about 3 years. The vesicles described herein, 20 including gas and gaseous precursor filled vesicles, may also be stable even under adverse conditions, such as temperatures and pressures which are above or below those experienced under normal ambient conditions.

The stability of the vesicles described herein may be attributable, at least in part, to the materials from which the vesicles are made, including, for example, the lipids, 25 polymers and/or proteins described above, and it is often not necessary to employ additional stabilizing materials, although it is optional and may be preferred to do so. Such additional stabilizing materials and their characteristics are described more fully hereinafter.

The materials from which the vesicles are constructed are preferably 30 biocompatible lipid, protein or polymer materials, and of these, the biocompatible lipids are preferred. In addition, because of the ease of formulation, including the capability of

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preparing vesicles immediately prior to administration, these vesicles may be conveniently made on site.

The biocompatible polymers useful as stabilizing materials for preparing the gas and gaseous precursor filled vesicles may be of natural, semi-synthetic (modified natural) or synthetic origin. As used herein, the term polymer denotes a compound comprised of two or more repeating monomeric units, and preferably 10 or more repeating monomeric units. The phrase semi-synthetic polymer (or modified natural polymer), as employed herein, denotes a natural polymer that has been chemically modified in some fashion. Exemplary natural polymers suitable for use in the present invention include

5 naturally occurring polysaccharides. Such polysaccharides include, for example, arabinans, fructans, fucans, galactans, galacturonans, glucans, mannans, xylans (such as, for example, inulin), levan, fucoidan, carrageenan, galatocarolose, pectic acid, pectins, including amylose, pullulan, glycogen, amylopectin, cellulose, dextran, dextrin, dextrose, polydextrose, pustulan, chitin, agarose, keratan, chondroitan, dermatan, hyaluronic acid,

10 alginic acid, xanthan gum, starch and various other natural homopolymer or heteropolymers, such as those containing one or more of the following aldoses, ketoses, acids or amines: erythrose, threose, ribose, arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose, erythrulose, ribulose, xylulose, psicose, fructose, sorbose, tagatose, mannitol, sorbitol, lactose, sucrose, trehalose, maltose,

15 cellobiose, glycine, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, glucuronic acid, gluconic acid, glucaric acid, galacturonic acid, mannuronic acid, glucosamine, galactosamine, and neuraminic acid, and naturally occurring derivatives thereof. Accordingly, suitable polymers include, for example, proteins, such as albumin. Exemplary semi-synthetic polymers include

20 carboxymethylcellulose, hydroxymethylcellulose, hydroxypropylmethylcellulose, methylcellulose, and methoxycellulose. Exemplary synthetic polymers suitable for use in the present invention include polyethylenes (such as, for example, polyethylene glycol, polyoxyethylene, and polyethylene terephthalate), polypropylenes (such as, for example, polypropylene glycol), polyurethanes (such as, for example, polyvinyl alcohol (PVA),

25 polyvinyl chloride and polyvinylpyrrolidone), polyamides including nylon, polystyrene, polylactic acids, fluorinated hydrocarbons, fluorinated carbons (such as, for example,

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polytetrafluoroethylene), and polymethylmethacrylate, and derivatives thereof. Methods for the preparation of vesicles which employ polymers as stabilizing compounds will be readily apparent to those skilled in the art, once armed with the present disclosure, when the present disclosure is coupled with information known in the art, such as that described 5 and referred to in Unger, U.S. Patent No. 5,205,290, the disclosures of which are hereby incorporated herein by reference, in their entirety.

Particularly preferred embodiments of the present invention may involve vesicles which comprise three components: (1) a neutral lipid, for example, a nonionic or zwitterionic lipid, (2) a negatively charged lipid, and (3) a lipid bearing a stabilizing 10 material, for example, a hydrophilic polymer. Preferably, the amount of the negatively charged lipid will be greater than about 1 mole percent of the total lipid present, and the amount of lipid bearing a hydrophilic polymer will be greater than about 1 mole percent of the total lipid present. Exemplary and preferred negatively charged lipids include phosphatidic acids. The lipid bearing a hydrophilic polymer will desirably be a lipid 15 covalently linked to the polymer, and the polymer will preferably have a weight average molecular weight of from about 400 to about 100,000. Suitable hydrophilic polymers are preferably selected from the group consisting of polyethylene glycol (PEG), polypropylene glycol, polyvinylalcohol, and polyvinylpyrrolidone and copolymers thereof, with PEG polymers being preferred. Preferably, the PEG polymer has a molecular weight of from 20 about 1000 to about 7500, with molecular weights of from about 2000 to about 5000 being more preferred. The PEG or other polymer may be bound to the lipid, for example, DPPE, through a covalent bond, such as an amide, carbamate or amine linkage. In addition, the PEG or other polymer may be linked to a targeting ligand, or other phospholipids, with a covalent bond including, for example, amide, ester, ether, thioester, thioamide or disulfide 25 bonds. Where the hydrophilic polymer is PEG, a lipid bearing such a polymer will be said to be "pegylated." In preferred form, the lipid bearing a hydrophilic polymer may be DPPE-PEG, including, for example, DPPE-PEG5000, which refers to DPPE having a polyethylene glycol polymer of a mean weight average molecular weight of about 5000 attached thereto (DPPE-PEG5000). Other suitable pegylated lipids include, for example, 30 distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG), including DSPE-

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PEG5000, dipalmitoyl-glycero-succinate-polyethylene glycol (DPGS-PEG), stearyl-polyethylene glycol and cholesteryl-polyethylene glycol.

In certain preferred embodiments of the present invention, the lipid compositions may include about 77.5 mole % DPPC, 12.5 mole % of DPPA, and 10 mole % of DPPE-PEG5000. Also preferred are compositions which comprise about 80 to about 90 mole % DPPC, about 5 to about 15 mole % DPPA and about 5 to about 15 mole % DPPE-PEG5000. Especially preferred are compositions which comprise DPPC, DPPA and DPPE-PEG5000 in a mole % ratio of 82:10:8, respectively. DPPC is substantially neutral, since the phosphatidyl portion is negatively charged and the choline portion is positively charged. Consequently, DPPA, which is negatively charged, may be added to enhance stabilization in accordance with the mechanism described above. DPPE-PEG provides a pegylated material bound to the lipid membrane or skin of the vesicle by the DPPE moiety, with the PEG moiety free to surround the vesicle membrane or skin, and thereby form a physical barrier to various enzymatic and other endogenous agents in the body whose function is to degrade such foreign materials. The DPPE-PEG may provide more vesicles of a smaller size which are safe and stable to pressure when combined with other lipids, such as DPPC and DPPA, in the given ratios. It is also theorized that the pegylated material, because of its structural similarity to water, may be able to defeat the action of the macrophages of the human immune system, which would otherwise tend to surround and remove the foreign object. The result is an increase in the time during which the stabilized vesicles may function as diagnostic imaging contrast media.

The vesicle compositions may be prepared from other materials, in addition to the materials described above, provided that the vesicles so prepared meet the stability and other criteria set forth herein. These materials may be basic and fundamental, and form the primary basis for creating or establishing the stabilized gas and gaseous precursor filled vesicles. On the other hand, they may be auxiliary, and act as subsidiary or supplementary agents which can enhance the functioning of the basic stabilizing material or materials, or contribute some desired property in addition to that afforded by the basic stabilizing material.

However, it is not always possible to determine whether a given material is a basic or an auxiliary agent, since the functioning of the material in question is

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determined empirically, for example, by the results produced with respect to producing stabilized vesicles. As examples of how these basic and auxiliary materials may function, it has been observed that the simple combination of a biocompatible lipid and water or saline when shaken will often give a cloudy solution subsequent to autoclaving for 5 sterilization. Such a cloudy solution may function as a contrast agent, but is aesthetically objectionable and may imply instability in the form of undissolved or undispersed lipid particles. Cloudy solutions may be also undesirable where the undissolved particulate matter has a diameter of greater than about 7 μm , and especially greater than about 10 μm . Manufacturing steps, such as sterile filtration, may also be problematic with solutions 10 which contain undissolved particulate matter. Thus, propylene glycol may be added to remove this cloudiness by facilitating dispersion or dissolution of the lipid particles. The propylene glycol may also function as a wetting agent which can improve vesicle formation and stabilization by increasing the surface tension on the vesicle membrane or skin. It is possible that the propylene glycol can also function as an additional layer that 15 may coat the membrane or skin of the vesicle, thus providing additional stabilization. As examples of such further basic or auxiliary stabilizing materials, there are conventional surfactants which may be used; see D'Arrigo U.S. Patents Nos. 4,684,479 and 5,215,680.

Additional auxiliary and basic stabilizing materials include such agents as peanut oil, canola oil, olive oil, safflower oil, corn oil, or any other oil commonly known 20 to be ingestible which is suitable for use as a stabilizing compound in accordance with the teachings herein. Various auxiliary and basic stabilizing materials are disclosed, for example, in U.S. application Serial No. 08/444,574, filed May 19, 1995, the disclosures of which are incorporated herein by reference, in their entirety.

In addition, compounds used to make mixed micelle systems may be 25 suitable for use as basic or auxiliary stabilizing materials, and these include, for example, lauryltrimethylammonium bromide (dodecyl-), cetyltrimethylammonium bromide (hexadecyl-), myristyltrimethylammonium bromide (tetradecyl-), alkyldimethylbenzylammonium chloride (where alkyl is C₁₂, C₁₄ or C₁₆), benzylidemethyldodecylammonium bromide/chloride, benzylidemethyl hexadecylammonium bromide/chloride, 30 benzylidemethyl tetradecylammonium bromide/chloride, cetyltrimethylammonium bromide/chloride, or cetylpyridinium bromide/chloride.

It has also been found that the gas and gaseous precursor filled vesicles used in the present invention may be controlled according to size, solubility and heat stability by choosing from among the various additional or auxiliary stabilizing materials described herein. These materials can affect these parameters of the vesicles, especially

5 vesicles formulated from lipids, not only by their physical interaction with the membranes, but also by their ability to modify the viscosity and surface tension of the surface of the gas and gaseous precursor filled vesicle. Accordingly, the gas and gaseous precursor filled vesicles used in the present invention may be favorably modified and further stabilized, for example, by the addition of one or more of a wide variety of (a) viscosity modifiers,

10 including, for example, carbohydrates and their phosphorylated and sulfonated derivatives; polyethers, preferably with molecular weight ranges between 400 and 100,000; and di- and trihydroxy alkanes and their polymers, preferably with molecular weight ranges between 200 and 50,000; (b) emulsifying and/or solubilizing agents including, for example, acacia, cholesterol, diethanolamine, stearates, including glyceryl monostearate, lanolin alcohols,

15 lecithin, mono- and di-glycerides, mono-ethanolamine, oleic acid, oleyl alcohol, poloxamer, for example, poloxamer 188, poloxamer 184, and poloxamer 181, polyoxyethylene 50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether, polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80, propylene glycol diacetate, propylene glycol monostearate, sodium lauryl

20 sulfate, sodium stearate, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan monostearate, stearic acid, trolamine, palmitates, and emulsifying wax;

(c) suspending and/or viscosity-increasing agents, including, for example, acacia, agar, alginic acid, aluminum mono-stearate, bentonite, magma, carbomer 934P, carboxymethylcellulose, calcium and sodium and sodium 12, carrageenan, cellulose,

25 dextran, gelatin, guar gum, locust bean gum, veegum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium-aluminum-silicate, Zeolites®, methylcellulose, pectin, polyethylene oxide, povidone, propylene glycol alginate, silicon dioxide, sodium alginate, tragacanth, xanthan gum, α-d-gluconolactone, glycerol and mannitol; (d) synthetic suspending agents, such as polyethylene glycol (PEG),

30 polyvinylpyrrolidone (PVP), polyvinylalcohol (PVA), polypropylene glycol (PPG), and

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polysorbate; and (e) tonicity raising agents which stabilize and add tonicity, including, for example, sorbitol, mannitol, trehalose, sucrose, propylene glycol and glycerol.

As noted above, the compositions of the present invention further comprise a targeting ligand. Generally speaking, materials which may be employed as targeting ligands include, for example, proteins, including antibodies, glycoproteins and lectins, peptides, polypeptides, saccharides, including mono- and polysaccharides, vitamins, steroids, steroid analogs, hormones, cofactors, bioactive agents, and genetic material, including nucleosides, nucleotides and polynucleotides.

The targeting ligands which may be incorporated in the compositions of the present invention are preferably substances which are capable of targeting receptors and/or tissues *in vivo*. With respect to the targeting of tissue, as noted above, the targeting ligands are desirably capable of targeting heart tissue, including myocardial cells, and membranous tissues, including endothelial and epithelial cells. In the case of receptors, the targeting ligands are desirably capable of targeting GPIIbIIIa receptors. It is contemplated that preferred targeting ligands for use in targeting tissues and/or receptors, including the tissues and receptors exemplified above, are selected from the group consisting of proteins, peptides, saccharides, steroids, steroid analogs, bioactive agents and genetic material, including, for example, antibodies, glycoproteins and lectins, with peptides being preferred. An example of a protein which may be preferred for use as a targeting ligand is Protein A, which is protein that is produced by most strains of *Staphylococcus aureus*. Protein A is commercially available, for example, from Sigma Chemical Co. (St. Louis, MO). Protein A may then be used for binding a variety of IgG antibodies. Generally speaking, peptides which are particularly useful as targeting ligands include natural, modified natural, or synthetic peptides that incorporate additional modes of resistance to degradation by vascularly circulating esterases, amidases, or peptidases. One very useful method of stabilization of peptide moieties incorporates the use of cyclization techniques. As an example, the end-to-end cyclization whereby the carboxy terminus is covalently linked to the amine terminus via an amide bond may be useful to inhibit peptide degradation and increase circulating half-life. Additionally, a side chain-to-side chain cyclization is also particularly useful in inducing stability. In addition, an end-to-side chain cyclization may be a useful modification as well. In addition, the

substitution of an L-amino acid for a D-amino acid in a strategic region of the peptide may offer resistance to biological degradation. Suitable targeting ligands, and methods for their preparation, will be readily apparent to one skilled in the art, once armed with the disclosure herein.

- 5 In connection with the targeting of endothelial cells, suitable targeting ligands include, for example, one or more of the following: growth factors, including, for example, basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), transforming growth factor-alpha (TGF- α), transforming growth factor-beta (TGF- β), platelet-derived endothelial cell growth factor (PD-ECGF) vascular endothelial growth factor (VEGF) and human growth factor (HGF); angiogenin; tumor necrosis factors, including tumor necrosis factor-alpha (TNF- α) and tumor necrosis factor-beta (TNF- β); copper-containing polyribonucleotide angiotropin with a molecular weight of about 4,500, as well as low molecular weight non-peptide angiogenic factors, such as 1-butyryl glycerol; the prostaglandins, including, for example, prostaglandin E₁ (PGE₁) and
- 10 prostaglandin E₂ (PGE₂); nicotinamide; adenosine; dipyridamole; dobutamine; hyaluronic acid degradation products, such as, for example, degradation products resulting from hydrolysis of β linkages, including hyalobiuronic acid; angiogenesis inhibitors, including, for example, collagenase inhibitors; minocycline; medroxyprogesterone; chitin chemically modified with 6-O-sulfate and 6-O-carboxymethyl groups; angiostatic steroids, such as
- 15 tetrahydrocortisol; and heparin, including fragments of heparin, such as, for example, fragments having a molecular weight of about 6,000, admixed with steroids, such as, for example, cortisone or hydrocortisone; angiogenesis inhibitors, including angioinhibin (AGM-1470 - an angiostatic antibiotic); platelet factor 4; protamine; sulfated polysaccharide peptidoglycan complexes derived from the bacterial wall of an *Arthobacter*
- 20 species; fungal-derived angiogenesis inhibitors, such as fumagillin derived from *Aspergillus fumigatus*; D-penicillamine; gold thiomalate; thrombospondin; vitamin D₃ analogues, including, for example, 1- α , 25-dihydroxyvitamin D₃ and a synthetic analogue 22-oxa-1- α , 25-dihydroxyvitamin D₃; α -interferon; cytokines, such as the interleukins, including, for example, interleukin-1 (IL-1), interleukin-2 (IL-2), and interleukin-8 (IL-8);
- 25 granulocyte macrophage colony stimulating factor (GMCSF); heparin, including low molecular weight fragments of heparin or analogues of heparin; simple sulfated

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polysaccharides, such as cyclodextrins, including α -, β - and γ -cyclodextrin; tetradecasulfate; transferrin; ferritin; platelet factor 4; protamine; Gly-His-Lys complexed to copper; ceruloplasmin; (12R)-hydroxycicosatrienoic acid; okadaic acid; lectins; antibodies; CD11a/CD18; and Very Late Activation Integrin-4 (VLA-4).

5 Endothelial-leukocyte adhesion molecules (ELAM's) are antigens which are expressed by endothelial cells under conditions of stress which then facilitate the migration of the leukocytes across the endothelium lining the vasculature into the surrounding tissues. It is also the surprising discovery that these same endothelial-leukocyte adhesion molecules may be advantageously exploited as receptors for targeting of vesicles. These
10 endothelial cell adhesion molecules belong to a family known as selectins in which the known members, such as GMP-140, all participate in endothelial-leukocyte adhesion and include ELAM-1, LAM-1 and the granule membrane protein 140 (GMP-140) also known as platelet activation-dependent granule-external membrane protein (PADGEM), VCAM-1/INCAM-110 (Vascular Adhesion Molecule/Inducible Adhesion Molecule) and ICAM-1
15 (Intercellular Adhesion Molecule). The cadherin family of cell adhesion molecules may also be used as targeting ligands, including for example, the E-, N-, and P-cadherins, cadherin-4, cadherin-5, cadherin-6, cadherin-7, cadherin-8, cadherin-9, cadherin-10, and cadherin-11; and most preferably cadherin C-5. Further, antibodies directed to cadherins, such as, for example, the monoclonal antibody Ec6C10, may be used to recognize
20 cadherins expressed locally by specific endothelial cells.

A wide variety of different targeting ligands can be selected to bind to the cytoplasmic domains of the ELAM molecules. Targeting ligands in this regard may include lectins, a wide variety of carbohydrate or sugar moieties, antibodies, antibody fragments, Fab fragments, such as, for example, Fab'2, and synthetic peptides, including,
25 for example, Arginine-Glycine-Aspartic Acid (R-G-D) which may be targeted to wound healing. While many of these materials may be derived from natural sources, some may be synthesized by molecular biological recombinant techniques and others may be synthetic in origin. Peptides may be prepared by a variety of different combinatorial chemistry techniques as are now known in the art. Targeting ligands derived or modified
30 from human leukocyte origin, such as CD11a/CD18, and leukocyte cell surface glycoprotein (LFA-1), may also be used as these are known to bind to the endothelial cell

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receptor ICAM-1. The cytokine inducible member of the immunoglobulin superfamily, VCAM-1, which is mononuclear leukocyte-selective, may also be used as a targeting ligand. VLA-4, derived from human monocytes, may be used to target VCAM-1.

5 Antibodies and other targeting ligands may be employed to target endoglin, which is an endothelial cell proliferation marker. Endoglin is upregulated on endothelial cells in miscellaneous solid tumors. A targeting ligand which may be used to target endoglin is the antibody TEC-11. R.E. Thorpe and F.J. Burrows, *Breast Cancer Research and Treatment*, Vol. 36, pp. 237-51 (1995).

Endothelial cell activation in the setting of atherosclerosis is used in this

10 invention to target the compositions to regions of arteriosclerosis including, for example, atherosclerotic plaque. One such target that can be used is the inducible mononuclear leukocyte endothelial adhesion molecule recognized by Rb1/9 as an ATHERO-ELAM. The monoclonal antibodies, H4/18 and H18/7, may be used to target endothelial cell surface antigens which are induced by cytokine mediators. As a preferred embodiment of

15 this invention, gas filled vesicles are targeted to atherosclerotic plaque to non-invasively detect diseased blood vessels before severe damage has occurred, for example, prior to stroke or myocardial infarction, so that appropriate medical or surgical intervention may be implemented. ATHERO-ELAM is a preferred target and ligands, such as antibodies, peptides, or lectins or combinations thereof may be used to target this cell surface epitope

20 expressed on endothelial cells in the context of atherosclerosis. Alternatively, lipoproteins or lipoprotein fragments derived from low or high density lipoprotein proteins may be used as targeting ligands. Additionally, cholesterol may be used to target the endothelial cells and localize the lipids, vesicles, and the like, to regions of atherosclerotic plaque. In embodiments which involve the use of cholesterol as a targeting ligand, the cholesterol is

25 preferably unmodified (non-derivatized) with other chemical groups, moieties, ligands, and the like.

A targeting ligand directed toward thrombotic material in the plaque may be used to differentiate between active and inactive regions of atherosclerotic plaque.

Active plaques in the process of generating thrombi are more dangerous as these plaques

30 may ultimately occlude a vessel or result in emboli. In this regard, in addition to low molecular weight heparin fragments, other targeting ligands, such as, for example, anti-

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fibrin antibody, tissue plasminogen activator (t-PA), anti-thrombin antibody and fibrin antibodies directed to platelet activation factors, may be used to target active plaque with evolving clots. Most preferred targeting ligands are those which will target a plasma membrane associated GPIIbIIIa in activated platelets in addition to targeting P-selectin,

5 and an antibody or associated antibody fragment directed to GPIIbIIIa. The present invention is also useful for detecting regions of acute myocardial infarction. Conveniently, by attaching anti-myosin (particularly cardiomyosin) antibody or anti-actin antibodies to the lipids, polymers or stabilizing materials, infarcted myocardium may be detected by the methods of the present invention. For targeting to granulation tissue (healing wounds),

10 many of the above targeting ligands may be useful. The wound healing tripeptide, arginine-glycine-aspartic acid (RGD), may also be used as a targeting ligand in this regard.

As with the endothelial cells discussed above, a wide variety of peptides, proteins and antibodies may be employed as targeting ligands for targeting epithelial cells. Preferably, a peptide, including synthetic, semi-synthetic or naturally-occurring peptides,

15 with high affinity to the epithelial cell target receptor may be selected, with synthetic peptides being more preferred. In connection with these preferred embodiments, peptides having from about 5 to about 15 amino acid residues are preferred. Antibodies may be used as whole antibody or antibody fragments, for example, Fab or Fab'2, either of natural or recombinant origin. The antibodies of natural origin may be of animal or human origin,

20 or may be chimeric (mouse/human). Human recombinant or chimeric antibodies are preferred and fragments are preferred to whole antibody.

Examples of monoclonal antibodies which may be employed as targeting ligands in the present compositions include CALAM 27, which is formed by immunizing BALB/c mice with whole human squamous cell carcinoma of the tongue and forming hybridomas by crossing extracted spleen cells with those of an NS1 syngeneic myeloma cell line. Gioanni, J. et al., *Cancer Research*, Vol. 47, pp. 4417-4424 (1987). CALAM 27 is directed to surface epitopes of both normal and malignant epithelial cells. Normal lymph nodes generally do not contain cells expressing these epitopes. See *Cancer Research*, Vol. 47, pp. 4417-4424 (1987). Accordingly, lipid and/or vesicle compositions comprising this antibody can be used to target metastases in the lymph nodes. The monoclonal antibody 3C2 may be employed as a targeting ligand for targeting malignant

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epithelial cells of serious ovarian carcinoma and endometrioid carcinoma. Another exemplary targeting ligand is Mab 4C7 (*see Cancer Research*, Vol. 45, 2358-2362, 1985), which may be used to target mucinous carcinoma, endometriod carcinoma and mesonephroid carcinoma. For targeting squamous cell carcinoma in head and neck cancer,
5 Mab E48 (*Biological Abstract*, Vol. 099 Issue. 066 Ref. 082748) may be used as a targeting ligand. For targeting malignant melanoma, the monoclonal antibody 225.28s (*Pathol. Biol.*, Vol. 38 (8), pp. 866-869, 1990) may be employed.

Targeting ligands may be selected for targeting antigens, including antigens associated with breast cancer, such as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor, erbB2/HER-2 and tumor associated carbohydrate antigens (*Cancer*,
10 Vol. 74 (3) pp. 1006-12 (1994)). CTA 16.88, homologous to cytokeratins 8, 18 and 19, is expressed by most epithelial-derived tumors, including carcinomas of the colon, pancreas, breast, ovary and lung. Thus, antibodies directed to these cytokeratins, such as 16.88 (IgM) and 88BV59 (IgG3k), which recognize different epitopes on CTA 16.88 (*Semin.*
15 *Nucl. Med.*, Vol. 23 (2), pp. 165-79 (1993)), may be employed as targeting ligands. For targeting colon cancer, anti-CEA IgG Fab' fragments may be employed as targeting ligands. Chemically conjugated bispecific anti-cell surface antigen, anti-hapten Fab'-Fab antibodies may also be used as targeting ligands. The MG series monoclonal antibodies
may be selected for targeting, for example, gastric cancer (*Chin. Med. Sci. J.*, Vol. 6 (1),
20 pp. 56-59 (1991).

There are a variety of cell surface epitopes on epithelial cells for which targeting ligands may be selected. For example, the protein human papilloma virus (HPV) has been associated with benign and malignant epithelial proliferations in skin and mucosa. Two HPV oncogene proteins, E6 and E7, may be targeted as these may be expressed in certain epithelial derived cancers, such as cervical carcinoma. *See Curr. Opin. Immunol.* Vol. 6 (5), pp. 746-54 (1994). Membrane receptors for peptide growth factors (PGF-R), which are involved in cancer cell proliferation, may also be selected as tumor antigens. *Anticancer Drugs*, Vol. 5 (4), pp. 379-93 (1994). Also, epidermal growth factor (EGF) and interleukin-2 may be targeted with suitable targeting ligands, including peptides, which bind these receptors. Certain melanoma associated antigens (MAA), such as epidermal growth factor receptor (EGFR) and adhesion molecules (*Tumor Biol.*, Vol. 15

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(4), pp. 188-202 (1994)), which are expressed by malignant melanoma cells, can be targeted with the compositions provided herein. The tumor associated antigen FAB-72 on the surface of carcinoma cells may also be selected as a target.

- A wide variety of targeting ligands may be selected for targeting
- 5 myocardial cells. Exemplary targeting ligands include, for example, anticardiomysin antibody, which may comprise polyclonal antibody, Fab'2 fragments, or be of human origin, animal origin, for example, mouse origin, or of chimeric origin. Additional targeting ligands include dipyridamole; digitalis; nifedipine; apolipoprotein; low density lipoproteins (LDL), including α -LDL, vLDL and methyl LDL; ryanodine; endothelin;
- 10 complement receptor type 1; IgG Fc; beta 1-adrenergic; dihydropyridine; adenosine; mineralocorticoid; nicotinic acetylcholine and muscarinic acetylcholine; antibodies to the human alpha 1A-adrenergic receptor; bioactive agents, such as drugs, including the alpha 1-antagonist prazosin; antibodies to the anti-beta-receptor; drugs which bind to the anti-beta-receptor; anti-cardiac RyR antibodies; endothelin-1, which is an endothelial cell-
- 15 derived vasoconstrictor peptide that exerts a potent positive inotropic effect on cardiac tissue (endothelin-1 binds to cardiac sarcolemmal vesicles); monoclonal antibodies which may be generated to the T cell receptor alpha-beta receptor and thereby employed to generate targeting ligands; the complement inhibitor sCR1; drugs, peptides or antibodies which are generated to the dihydropyridine receptor; monoclonal antibodies directed
- 20 towards the antiinterleukin 2 receptor may be used as targeting ligands to direct the present compositions to areas of myocardial tissue which express this receptor and which may be up-regulated in conditions of inflammation; cyclosporine for directing similarly the compositions to areas of inflamed myocardial tissue; methylisobutyl isonitrile; lectins which bind to specific sugars on membranes of cardiac myocytes and cardiac endothelial
- 25 cells; adrenomedullin (ADM), which is an endogenous hypotensive and vasorelaxing peptide; atrial natriuretic peptide (ANP); C-type natriuretic peptide (CNP), which is a 22 amino acid peptide of endothelial cell origin and is structurally related to atrial natriuretic peptide but genetically distinct, and possesses vasoactive and antimitogenic activity; vasonatrin peptide (VNP) which is a chimera of atrial natriuretic peptide (ANP) and C-
- 30 type natriuretic peptide (CNP) and comprises 27 amino acids; thrombin; endothelium-derived relaxing factor (EDRF); neutral endopeptidase 1 (NEP- 1); competitive inhibitors

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to EDRF, including, for example, NG-monomethyl-L-arginine (L-NMMA); potassium channel antagonists, such as charybdotoxin and glibenclamide; antiheart antibodies, which may be identified in patients with idiopathic dilated cardiomyopathy but which preferably do not elicit cytolysis in the myocardium; antibodies directed against the adenine nucleotide translocator, the branched-chain keto acid dehydrogenase or cardiac myosin; specific antagonists for the endothelin-A receptor, which may be referred to as BQ-123; and antibodies to the angiotensin II receptor.

Two of the major antigens of heart sarcolemmal are calcium binding glycoproteins which copurify with the dihydropyridine receptor. Antisera may be raised, including polyclonal or monoclonal antibodies, against purified sarcolemma. These antibodies may also be employed as targeted ligands. Purified fractions of the calcium binding glycoproteins may be isolated from the plasma membranes of the sarcolemma and then used to generate antibodies. ANP, which, as noted above, may be used as a targeting ligand, can be obtained from cultures of human aortic endothelial cells. ANP is generally localized in endothelium, but also may localize to the endothelial or myocardial tissue. ANP may be prepared, for example, using recombinant techniques, as well as by synthesis of the peptide using peptide synthesis techniques well known to those skilled in the art. It is also possible to use an antibody, either polyclonal or monoclonal, directed towards ANP. Similarly, a peptide directed to ANP may be used for targeting endothelial and/or myocardial cells. Both the β and α forms of atrial natriuretic factor may be used as potential targeting ligands for directing the present compositions to myocardial tissue.

A wide variety of targeting ligands may be employed to direct the present lipid compositions, and particularly vesicle compositions, to the GPIIbIIIa receptor. In preferred form, the targeting ligand exhibits a binding affinity (K_d) to the GPIIbIIIa receptor of no greater than about 10^{-3} molar. More preferably, the targeting ligand exhibits a binding affinity (K_d) to the GPIIbIIIa receptor of less than about 10^{-3} molar such as, for example, binding affinities ranging from about 10^{-9} molar to less than about 10^{-3} molar, and all combinations and subcombinations of ranges of binding affinities therein. Even more preferably, the targeting ligand exhibits a binding affinity (K_d) to the GPIIbIIIa receptor of from about 10^{-7} molar to about 10^{-5} molar, with a binding affinity of about 10^{-6} molar being especially preferred.

Compositions which are directed to the GPIIbIIIa receptor are highly useful for targeting vascular thromboses or clots, and are useful for diagnosing, as well as treating such clots. In certain preferred embodiments, the targeting ligand is a peptide containing the sequence Arg-Gly-Asp (RGD). In other preferred embodiments, the targeting ligand is 5 a peptide containing the sequence Ala-Gly-Asp (AGD) such as, for example, peptides containing the sequence Lys-Gln-Ala-Gly-Asp-Val (KQAGDV) which is the γ -carboxy sequence of fibrinogen.

Included among the targeting ligands which are suitable for use in the compositions and methods of the present invention are peptides such as Arg-Gly-Asp-Ser 10 (RGDS), Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), and Gly-Pro-Arg-Pro (GPRP).

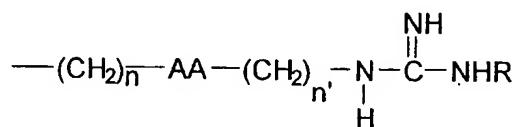
Pentapeptides containing the sequence Arg-Gly-Asp (RGD) are also useful including, for example, G4120, which is a cyclic peptide containing the amino acid sequence Arg-Gly-Asp (RGD). Also useful are peptides derived from human coagulation Factor XIII A including, for example, fragments such as

15 NKLIVRRGQSFYVQIDFSRPYDPRRLFLRVEYVIGRYPQENKGTYIPVPIVSELQSG
KWGAKIVMREDRSVRLSIQSSPKCIVGKFRMYVAVWTPYGVLRTRNPETDTYIL
FNPWCEDDAVYLDNEKEREELYVLNDIGVIFYGEVNDIKTRSWSYGQF-R' where R'
is -CONH₂ or -NH₂. In addition, peptides which are fragments of the Factor XIII A
fragment, which include in their sequence the sequence
20 NKLIVRRGOSFYVQIDFSRPYDPRRD or
DDAVYLDNEKEREELYVLNDIGVIFYGEVNDIKTRSWSYGQF.

Additional peptides which may be useful as targeting ligands for targeting the GPIIbIIIa receptor include, for example, peptides comprising the tripeptide sequence of arginine-tyrosine-aspartic acid (Arg-Tyr-Asp; also abbreviated RGD), linked from amino-25 to-carboxy-terminus and which may bind to the GPIIbIIIa binding region on activated platelets. Exemplary of such peptides include, for example, peptides of the general formula R¹-(X¹)_n-Arg-Tyr-Asp-(Y)_o-(X²)_m-R², wherein each of X¹, X² and Y may independently be one or more amino acid residues while, in certain cases, it is preferred that Y is other than a serine or alanine residue, and each of m, n and o is independently 0 30 or 1, provided, in certain cases, that when m is 1, then o is 1, and R¹ is a protected or unprotected terminal amino group and R² is a protected or unprotected terminal carboxy

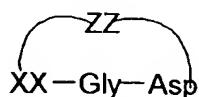
group. In a preferred embodiment, X¹ is the peptide Ala-Arg-Arg-Ser-Ser-Pro-Ser-Tyr-Tyr and X² is the peptide Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr. Useful peptides include Arg-Ser-Pro-Ser-Tyr-Tyr-Arg-Tyr-Asp-Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-Met-Asp-Tyr and Ala-Arg-Arg-Ser-Pro-Ser-Tyr-Tyr-Arg-Tyr-Asp-Gly-Ala-Gly-Pro-Tyr-Tyr-Ala-
5 Met-Asp-Tyr.

Synthetic compounds which combine a natural amino acid sequence with synthetic amino acids can also be used as the targeting ligand, such as a fibrinogen receptor antagonist compound which comprises the sequence XX-Gly-Asp, wherein XX is a synthetic α -amino acid containing a linear side chain, such as



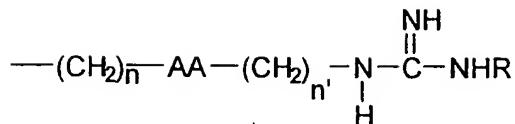
10 wherein n + n' is 3; AA is a single bond; and R is phenyl or benzyl; or $\text{---}(\text{CH}_2)_n\text{---AA---}(\text{CH}_2)_{n'}\text{---NHR}$, wherein n is an integer of 1 to 4; n' is an integer of 2 to 4; AA is oxygen, sulfur or a single bond; and R is H, C₁₋₆ alkyl, optionally substituted aryl, optionally substituted arylmethyl or optionally substituted cycloalkyl, provided, in certain cases, that when AA is a single bond and R is H, then n + n' is other than 3 or 4.

15 Another such compound comprises a fibrinogen receptor antagonist of the formula:



wherein XX is a synthetic α -amino acid containing a linear side chain having the formula

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wherein n + n' is 3; AA is a single bond; and R is phenyl or benzyl; or

—(CH₂)_n—AA—(CH₂)_{n'}—NHR, wherein n is an integer of 1 to 4; n' is an integer of 2 to 4; AA is oxygen, sulfur or a single bond; and R is H, C₁₋₆ alkyl, optionally substituted cycloalkyl, provided that, in certain cases, when AA is a single bond and R is H, then n +

5 n' is other than 3 or 4, and ZZ is a sequence of 1 to 4 optionally substituted amino acids.

Other useful peptides for use as targeting ligands include, for example, "Elegantin", which has the following sequence: Gly-Glu-Glu-Cys-Asp-Cys-Gly-Ser-Pro-Glu-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr-Cys-Lys-Leu-Arg-Pro-Gly-Ala-Gln-Cys-Ala-Asp-Gly-Leu-Cys-Cys-Asp-Gln-Cys-Arg-Phe-Lys-R-R'-Arg-Thr-Ile-Cys-Arg-Arg-Ala-10 Arg-Gly-Asp-Asn-Pro-Asp-Asp-Arg-Cys-Thr-Gly-Gln-Ser-Ala-Asp-Cys-Pro-Arg-Asn-Gly-Tyr, wherein each of R and R' is independently any amino acid; "Albolabrin", which has the following sequence: Glu-Ala-Gly-Glu-Asp-Cys-Asp-Cys-Gly-Ser-Pro-Ala-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr-Cys-Lys-Leu-Leu-Pro-Gly-Ala-Gln-Cys-Gly-Glu-Gly-Leu-Cys-Cys-Asp-Gln-Cys-Ser-Phe-Met-Lys-Lys-Gly-Thr-Ile-Cys-Arg-Arg-Ala-Arg-15 Gly-Asp-Asp-Leu-Asp-Asp-Tyr-Cys-Asn-Gly-Ile-Ser-Ala-Gly-Cys-Pro-Arg-Asn-Pro-Leu-His-Ala; "Batroxostatin", which has the following sequence: Glu-Ala-Gly-Glu-Glu-Cys-Asp-Cys-Gly-Thr-Pro-Glu-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr-Cys-Lys-Leu-Arg-Pro-Gly-Ala-Gln-Cys-Ala-Glu-Gly-Leu-Cys-Cys-Asp-Gln-Cys-Arg-Phe-Lys-Gly-Ala-Gly-Lys-Ile-Cys-Arg-Arg-Ala-Arg-Gly-Asp-Asn-Pro-Asp-Asp-Cys-Thr-Gly-Gln-Ser-Ala-20 Asp-Cys-Pro-Arg-Phe; and "Flavoridin", which has the following sequence: Gly-Gly-Glu-Cys-Asp-Cys-Gly-Ser-Pro-Glu-Asn-Pro-Cys-Cys-Asp-Ala-Ala-Thr-Cys-Lys-Leu-Arg-Pro-Gly-Ala-Gln-Cys-Ala-Asp-Gly-Leu-Cys-Cys-Asp-Gln-Cys-Arg-Phe-Lys-R-R'-Arg-Thr-Ile-Cys-Arg-Ile-Ala-Arg-Gly-Asp-Phe-Pro-Asp-Asp-Arg-Cys-Thr-Gly-Leu-Ser-Ala-Asp-Cys-Pro-Arg-R-Asn-Asp-Leu, wherein each of R and R' is independently any amino-25 acid.

Other ligands useful for targeting the GPIIbIIIa receptor include synthetic compounds, such as Ac-(D)Phe-Pro-boroArg and the cyclic peptidomimetic cyclo(D-2-

aminobutyrate-N-Methyl-L-Arginyl-Glycyl-L-Aspartyl-3-amino-methyl-benzoic acid) methanesulfonate salt. Peptides that can also be used include a library of hexapeptides flanked by cysteine residues (capable of forming cyclic disulfides) and cyclic, disulfide-bonded forms of peptides with the sequence Arg-Gly-Asp or Lys-Gly-Asp, as well as the 5 carboxyl-terminal derived peptide, REYVVMWK. Certain matrix glycoproteins such as Thrombospondin are also useful in this regard. Members of the serpin family of serine protease inhibitors, such as Plasminogen activator inhibitor type 1 (PAI-1) are other useful ligands. Other synthetic compounds which may be useful for targeting the GPIIbIIIa receptor include, for example, ticlopidine, clopidogrel, tirofiban, and abciximab and 10 analogs and derivatives thereof.

Generally speaking, it is preferred to employ as targeting ligands for the GPIIbIIIa receptor a peptide having from about 3 to about 20 amino acids, with peptides having from about 4 to about 15 amino acids being more preferred. Even more preferably, targeting ligands for the GPIIbIIIa receptor may comprise peptides having from about 4 to 15 about 8 amino acids, with peptides having from about 4 to about 6 amino acids or about 5 amino acids being still more preferred.

If desired, the peptides may be cyclized, for example, by (1) sidechain-to-sidechain covalent linkages, including, for example, by the formation of a disulfide linkage via the oxidation of two thiol containing amino acids or analogs thereof, including, for 20 example, cysteine or penicillamine; (2) end-to-sidechain covalent linkages, including, for example, by the use of the amino terminus of the amino acid sequence and a sidechain carboxylate group, such as, for example, a non-critical glutamic acid or aspartic acid group. Alternatively, the end-to-sidechain covalent linkage may involve the carboxylate terminus of the amino acid sequence and a sidechain amino, amidine, guanidine, or other 25 group in the sidechain which contains a nucleophilic nitrogen atom, such sidechain groups including, for example, lysine, arginine, homoarginine, homolysine, or the like; (3) end-to-end covalent linkages that are covalent amide linkages, or the like. Such processes are well known to those skilled in the art. The peptides may also be cyclized via the addition of flanking amino acids. For example, in the case of targeting ligands comprising the 30 tripeptide RGD, flanking amino acids may be added to form $(X)_n$ -RGD-(Y)_n where n is an integer of from about 1 to about 100 and X and Y may be any natural or synthetic amino

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acid and, with the proviso that at least one of the involved amino acids is cysteine or an analog such as penicillamine. These targeting ligands may be cyclized via cysteine sidechains with the cyclization occurring through disulfide bonds. Other modes of cyclization may involve end-to-end covalent linkages involving amino to carboxylate peptide bonds. In addition, X may be lysine and/or arginine and Y may be aspartate or glutamate with condensation of the side chain moieties to form a cyclic amide. Additional permutations include side chain group reactions with terminal amino or carboxyl groups.

In addition, "pseudocyclization" may be employed, in which cyclization occurs via non-covalent interactions, such as electrostatic interactions, which induces a folding of the secondary structure to form a type of cyclic moiety. It is contemplated that metal ions may aid the induction of a "pseudocyclic" formation. This type of pseudocyclic formation may be analogous to "zinc fingers." As known to one of ordinary skill in the art, zinc fingers involve the formation due to electrostatic interactions between a zinc ion (Zn_{2+}) and cysteine, penicillamine and/or homocysteine, of a region in the shape of a loop (the finger). In the case of homocysteine, the RGD sequence would reside at the tip of the finger. Of course, it is recognized that, in the context of the present invention, any type of stabilizing cyclization would be suitable as long the recognition and binding peptide ligand, such as, for example, RGD, maintains the proper conformation and/or topography to bind to the appropriate receptor in clots with a reasonable Michaelis-Menten constant (k_m) or binding constant. As used herein, the term "conformation" refers to the three-dimensional organization of the backbone of the peptide, peptoid, or pseudopeptide, and the term "topography", as used herein, refers to the three-dimensional organization of the sidechain of the peptide, peptoid, or pseudopeptide.

The targeting ligands may be incorporated in the present compositions in a variety of ways which would be apparent to the skilled artisan, once armed with the teachings of the present application. In certain preferred embodiments, including embodiments involving compositions which comprise lipid compounds, proteins, polymers and/or vesicles, the targeting ligands may be associated with the lipid compounds, proteins, polymers and/or vesicles covalently. In certain alternate preferred embodiments, including embodiments involving compositions which comprise lipid compounds, proteins, polymers and/or vesicles, there may be substantially no covalent

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association between the targeting ligands and other components of the compositions (*i.e.*, the targeting ligands may be free or unbound). For example, in embodiments involving lipid compounds, proteins, polymers and/or vesicles, the targeting ligand may be associated with the lipid compounds, proteins, polymers and/or vesicles non-covalently.

- 5 In other preferred embodiments in which the targeting ligands are free and/or unbound, the targeting ligands may be combined with the compositions such that the targeting ligands are neither covalently nor non-covalently associated with the other components of the compositions, including, for example, lipid compounds, proteins, polymers and/or vesicles.

10 The targeting ligands preferably promote targeting of the present compositions to the desired target, for example, receptors and/or tissues *in vivo*. Once targeted, the targeted compositions may desirably be located proximate the desired target. For example, it is contemplated that the compositions may be bound, for example, by physical and/or electrostatic interactions, to the desired target. It has been surprisingly and 15 unexpectedly found that free or unbound targeting ligand may promote targeting of the present compositions, such as vesicle compositions, including gas filled vesicles, to a desired target including, for example, blood clots. Without intending to be bound by any theory or theories of operation, it is contemplated that such binding may occur due to association of the targeting ligand with other components of the compositions, such as, 20 lipids, including lipids present in the surface of vesicles, for example, via van der Waal's interactions, electrostatic interactions or some other association process. In the case of preferred embodiments involving lipid compositions containing unbound targeting ligand, it is theorized also that it is possible that a functional group in the targeting ligand, such as, for example, an amino group, may spontaneously associate or bind directly to one of the 25 groups on the lipids, *e.g.*, a phosphatidic acid moiety, to form a phosphoroamidate bond.

Thus, in the case of lipid compositions, the targeting ligand may, if desired, be bound, such as via a covalent or non-covalent bond, to at least one of the lipids incorporated in the compositions. In the case of vesicles which are formulated from substances other than lipids, for example, clathrates, aerogels and albumin vesicles, the 30 targeting ligand may be bound covalently or non-covalently to one or more of the materials incorporated in the vesicle walls. Preferably, in the case of lipid compositions

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which comprise cholesterol, the targeting ligand is bound to the cholesterol substantially only non-covalently, and/or that the targeting ligand is bound covalently to a component of the composition, for example, another lipid, such as a phospholipid, other than the cholesterol. If desired, the targeting ligands may also be bound covalently and/or non-
5 covalently to other stabilizing materials, for example, biocompatible polymers, which may be present in the compositions.

In connection with targeting ligands which may be covalently bound to other components of the present compositions including, for example, lipids, polymers, proteins and/or vesicles, as well as other stabilizing materials, the targeting ligands may
10 preferably include at their distal end a functional group which may be useful, for example, in forming such covalent bonds. Examples of such distal functional groups include, for example, amino (-NH₂), hydroxy (-OH), carboxyl (-COOH), thiol (-SH), phosphate, phosphinate, sulfate and sulfinate. In the case of cyclized targeting ligands, the ligand preferably includes a functional group, such as amino, hydroxy, carboxyl, thiol, phosphate,
15 phosphinate, sulfate or sulfinate, through which the covalent linkage may be established and which is generally not critical for binding to the desired receptor, for example, GPIIbIIIa receptor. Also in the case of cyclized targeting ligands, the cyclization preferably exposes the backbone conformation and sidechain topography of the targeting ligand such as, for example, the sequences RGD or AGD, such as KQAGDV, to enable
20 binding of the ligand to the receptor, such as the GPIIbIIIa receptor.

Other suitable targeting ligands include the following compounds: Ac-Cys-Arg-Gly-Asp-Met-Phe-Gly-Cys-CONH₂; Ac-Cys-Arg-Gly-Asp-Met-Leu-Arg-Cys-CONH₂; Ac-Cys-Arg-Gly-Asp-Phe-Leu-Asn-Cys-CONH₂; Ac-Cys-Asn-Thr-Leu-Lys-Gly-Asp-Cys-CONH₂; Ac-Cys-Asn-Trp-Lys-Arg-Gly-Asp-Cys-CONH₂; and Ac-Cys-N-
25 methyl-Arg-Gly-Asp-Pen-CONH₂, where "Pen" refers to penicillamine (β,β -dimethylcysteine).

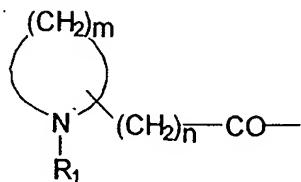
Other compounds which may be used as targeting ligands include peptides, or derivatives thereof, represented by the formula



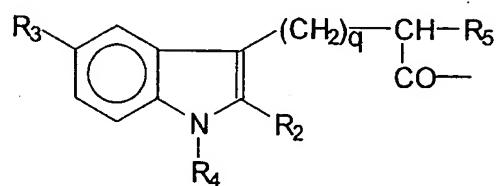
30 wherein:

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A is proline, thioproline, hydroxyproline, dehydroproline, 2-oxo-4-thiazolidine carboxylic acid, N-alkyl glycine or an amino acid derivative of the formula



tryptophan, or a tryptophan derivative of the formula



pyroglutamic acid or 2-azetidinone-4-carboxylic acid

- 5 B is serine, glycine, valine, alanine, threonine or β -alanine;
- C is an amino acid group having a hydrophobic functional group; and
- D is hydroxy or amino;

wherein:

- R₁ is hydrogen, $-(\text{CH}_2)_p\text{CH}_3$ or $-\text{CO}-(\text{CH}_2)_p\text{CH}_3$,
- 10 R₂ is hydrogen or alkyl;
- R₃ is hydrogen or alkoxy;
- R₄ is hydrogen or alkyl;
- R₅ is hydrogen, amino or acylamino;
- m is an integer of 2 to 5;
- 15 n is an integer of 0 to 2;
- p is an integer of 0 to 5; and
- q is an integer of 0 to 3.

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Another targeting ligand which may be suitable for use in connection with the present compositions is a peptide, a peptide derivative, or a salt thereof having the formula



5 wherein:

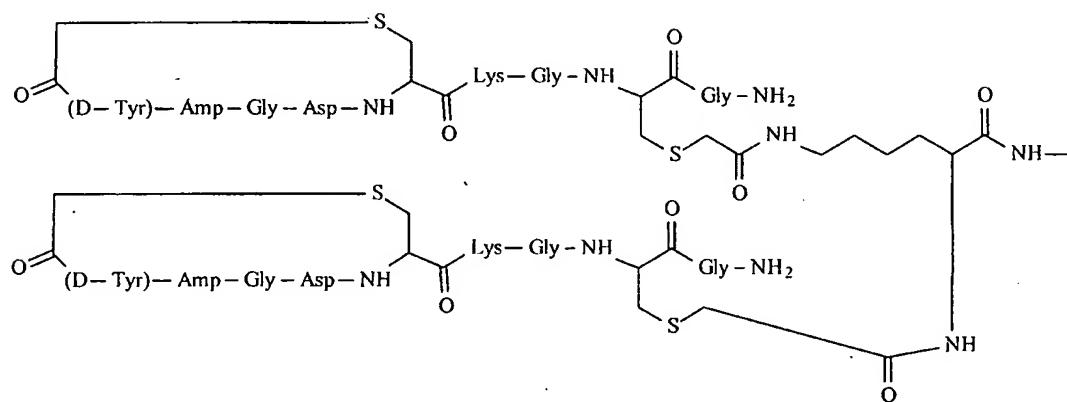
A is aromatic acid or hydroxyaromatic acid;

B is an amino acid;

C is an amino acid having a hydrophobic functional group; and

D is hydroxy or amino.

10 In the above compounds, examples of amino acids having hydrophobic functional groups in the definition of "C" are tryptophan and phenylalanine. Yet another targeting ligand which may be suitable for use in the compositions of the present invention is a peptide derivative having the formula

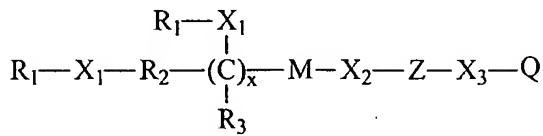


15 where "Amp" in the above structure is aminomethylphenyl acetic acid. Various peptides which would be suitable for use as a targeting ligand in connection with the present invention, especially for targeting GPIIbIIIa, are disclosed, for example, in Sato et al., U.S. Patent No. 5,498,601 and the following published European Patent Applications: 0 368 486 A2, 0 382 451 A2, and 0 422 938 B1, the disclosures of which are hereby incorporated 20 herein by reference, in their entirety. Other targeting ligands which may be used in the compositions of the present invention, in addition to those exemplified above, would be

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apparent to one of ordinary skill in the art, once armed with the present disclosure. Other suitable targeting ligands include, for example, conjugated peptides, such as, for example, glycoconjugates and lectins, which are peptides attached to sugar moieties. The compositions may comprise a single targeting ligand, as well as two or more different targeting ligands.

There are also provided, in accordance with the present invention, compounds having the formula



wherein:

- 10 x is 0 or 1;
- each X_1 is independently -O-, -S-, -SO-, -SO₂-, -NR₄-, -X₄-C(=X₅)-, -C(=X₅)-X₄- or -C(=X₅)-;
- each X_2 and X_3 is independently a direct bond, -R₅-X₄-C(=X₅)-, -R₅-C(=X₅)-X₄, -X₄-C(=X₅)-R₅-, -C(=X₅)-X₄-R₅-, -X₄-R₅-C(=X₅)-X₄-,
- 15 -R₅-X₄-C(=X₅)-R₅-C(=X₅)-X₄- or -R₅-C(=X₅)-X₄-R₅-X₄-C(=X₅)-;
- each X_4 is independently -O-, -NR₄- or -S-;
- each X_5 is independently O or S;
- M is a direct bond, -R₅-X₄-C(=X₅)-, -R₅-C(=X₅)-X₄-,
- R₅-X₄-(YX₅)P(=X₅)-X₄- or -X₄-(YX₅)P(=X₅)-X₄-R₅-,
- 20 Y is hydrogen or a pharmaceutically acceptable counter ion;
- Z is a direct bond or a hydrophilic polymer;
- Q is a targeting ligand or a precursor thereto;
- each R_1 is independently alkyl of 1 to about 50 carbons;
- each R_2 is independently a direct bond or alkylene of 1 to about 30 carbons;
- 25 each R_3 and R_4 is independently hydrogen or alkyl of 1 to about 10 carbons; and

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- each R_5 is independently a direct bond or alkylene of 1 to about 30 carbons; with the provisos that when three or more of X_1 , X_2 , X_3 , R_2 and M are direct bonds, then Z is a hydrophilic polymer, and when Z is a direct bond, then three or more of X_1 , X_2 , X_3 , R_2 and M are other than direct bonds.

In the above formula, it is intended that when any symbol appears more than once in a particular formula or substituent, its meaning in each instance is independent of the other.

- Also in the above formula, it is intended that when each of two or more adjacent symbols is defined as being a "direct bond" to provide multiple, adjacent direct bonds, the multiple and adjacent direct bonds devolve into a single direct bond. In addition, when x in the above formula is 0, then the groups R_2 and M are linked together, and the substituents attached directly to "C" (i.e., X_1-R_1 and R_3) are absent in the defined compound.
- In the above formula, each X_1 is independently $-O-$, $-S-$, $-SO-$, $-SO_2-$, $-NR_4-$, $-X_4-C(=X_5)-$, $-C(=X_5)-X_4-$ or $-C(=X_5)-$. In preferred embodiments, each X_1 is independently $-X_4-C(=X_5)-$, $-C(=X_5)-X_4-$ or $-C(=X_5)-$. More preferably, each X_1 is independently $-X_4-C(=X_5)-$ or $-C(=X_5)-X_4-$. Even more preferably, X_1 is $-C(=X_5)-X_4-$, for example, $-C(=O)-O-$ or $-C(=O)-NH-$.
- In the above formula, each X_2 and X_3 is independently a direct bond, $-R_5-X_4-C(=X_5)-$, $-R_5-C(=X_5)-X_4$, $-X_4-C(=X_5)-R_5-$, $-C(=X_5)-X_4-R_5-$, $-X_4-R_5-C(=X_5)-X_4-$, $-R_5-X_4-C(=X_5)-R_5-C(=X_5)-X_4-$ or $-R_5-C(=X_5)-X_4-R_5-X_4-C(=X_5)-$. In preferred embodiments, X_2 and X_3 are independently a direct bond, $-R_5-X_4-C(=X_5)-$, $-R_5-C(=X_5)-X_4$, $-X_4-C(=X_5)-R_5-$, $-C(=X_5)-X_4-R_5-$, $-X_4-R_5-C(=X_5)-X_4-$ or $-R_5-X_4-C(=X_5)-R_5-C(=X_5)-X_4-$, with $-R_5-C(=X_5)-X_4$ being more preferred. Even more preferably, X_2 is a direct bond, $-CH_2CH_2-C(=O)-NH-$ or $-CH_2CH_2NH-C(=O)-CH_2CH_2-C(=O)-NH-$ and X_3 is a direct bond, $-C(=O)-NH-$, $-NH-C(=O)-$, $-NH-C(=O)-CH_2$, $-NHCH_2-C(=O)-NH-$, $-NH-C(=O)-CH_2CH_2$ or $-(CH_2)_n-C(=O)-NH-$, where n is an integer ranging from about 1 to about 20 (and all combinations and subcombinations of ranges therein), preferably from about 1 to about 15, more preferably from about 1 to about 10.

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In the above formula, each X_4 is independently -O-, -NR₄- or -S-.

Preferably, X_4 is independently -O- or -NR₄-.

In the above formula, each X_5 is independently O or S. Preferably, X_5 is O.

In the above formula, M is a direct bond, -R₅-X₄-C(=X₅)-, -R₅-C(=X₅)-X₄-,

- 5 -R₅-X₄-(YX₅)P(=X₅)-X₄- or -X₄-(YX₅)P(=X₅)-X₄-R₅- . In certain preferred embodiments, M is -R₅-X₄-C(=X₅)- or -R₅-X₄-(YX₅)P(=X₅)-X₄-, with M more preferably being -CH₂O-C(=O) or -CH₂O-(HO)P(=O)-O-. In certain other preferred embodiments, M is -R₅-X₄-C(=X₅)- or -R₅-C(=X₅)-X₄- . In yet other preferred embodiments, M is -R₅-X₄-(YX₅)P(=X₅)-X₄- or -X₄-(YX₅)P(=X₅)-X₄-R₅- wherein at least one of X₄ or X₅ is S.

- 10 In still other preferred embodiments, M is a direct bond.

In the above formula, Z is a direct bond or a hydrophilic polymer.

- Preferred hydrophilic polymers are selected from the group consisting of polyalkyleneoxides, polyvinyl alcohol, polyvinylpyrrolidones, polyacrylamides, polymethacrylamides, polyphosphazenes, poly(hydroxyalkylcarboxylic acids) and 15 polyoxazolidines. More preferably, Z comprises a polyalkyleneoxide. Even more preferably, Z is a polyalkyleneoxide selected from the group consisting of polyethylene glycol and polypropylene glycol, with polyethylene glycol being still more preferred. In certain other preferred embodiments, Z is a hydrophilic polymer other than polyalkyleneoxides, including polyethylene glycol and polypropylene glycol. The 20 molecular weight of Z may vary, depending, for example, on the particular end-use of the compounds. Preferably, Z is a polymer having a molecular weight which ranges from about 100 to about 10,000, and all combinations and subcombinations of ranges therein. More preferably, Z is a polymer having a molecular weight of from about 1,000 to about 5,000. Also preferred are polymers which exhibit polydispersities ranging from greater 25 than about 1 to about 3, and all combinations and subcombinations of ranges therein. More preferably, Z is a polymer having a polydispersity of from greater than about 1 to about 2, with polydispersities of from greater than about 1 to about 1.5 being even more preferred, and polydispersities of from greater than about 1 to about 1.2 being still more preferred. In alternate preferred embodiments, Z is a direct bond.
- 30 In the above formula, Q is a targeting ligand or a precursor thereto. In embodiments where Q is a targeting ligand, Q is preferably selected from the various

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targeting ligands discussed in detail above. Thus, for example, Q preferably targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein GPIIbIIIa receptor. In certain preferred embodiments, Q targets cells or receptors selected from the group consisting of myocardial 5 cells, endothelial cells, epithelial cells and the glycoprotein GPIIbIIIa receptor. In addition, in embodiments where Q is a targeting ligand, Q is preferably selected from the group consisting of proteins, peptides, saccharides, steroids, steroid analogs, bioactive agents, and genetic material. In these latter embodiments, Q is preferably selected from the group consisting of proteins, peptides and saccharides. In embodiments where Q 10 comprises a peptide, Q preferably comprises the sequences Arg-Gly-Asp (RGD) or Ala-Gly-Asp (AGD), such as Lys-Gln-Ala-Gly-Asp-Val (KQAGDV); or Q may be a cyclic peptide, such as, for example, DMP 728. (See, e.g., Mousa et al., *Thrombosis Research*, Vol. 76 (2), pp. 109-119 (1994), the disclosures of which are hereby incorporated herein by reference, in their entirety.) In embodiments where Q comprises a 15 protein, Q is preferably Protein A. In embodiments where Q comprises a saccharide, Q is preferably a monosaccharide, with mannose and glucose being more preferred. In embodiments where Q is a precursor to a targeting ligand, Q preferably comprises a partially unsaturated or aromatic 5- to 7-membered monocyclic ring containing 1 or 2 N, O or S atoms, and more preferably a maleimide moiety or a pyridyl moiety.

20 In the above formula, each R₁ is independently alkyl which ranges from 1 to about 50 carbons, and all combinations and subcombinations of ranges therein, or alkenyl of from about 2 to about 50 carbons, and all combinations and subcombinations of ranges therein. Preferably, each R₁ is independently alkyl of greater than 1 to about 40 carbons. More preferably, each R₁ is independently alkyl of about 5 to about 30 carbons. 25 Even more preferably, each R₁ is independently alkyl of about 10 to about 30 carbons, with alkyl of from about 15 to about 28 carbons being yet more preferred. In certain preferred embodiments, R₁ is straight chain alkyl of about 15 to about 17 carbons. R₁ is also preferably fused cycloalkyl and/or cycloalkenyl. In these latter embodiments, R₁ is preferably certain fused cycloalkyl and/or alkenyl of from about 10 to 40 carbons, with 30 fused cycloalkyl and/or alkenyl of from about 20 to 30 carbons being more preferred. A particularly preferred fused cycloalkyl and/or alkenyl group is cholesterol.

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In the above formula, each R₂ is independently a direct bond or alkylene which ranges from 1 to about 30 carbons, and all combinations and subcombinations of ranges therein. Preferably, each R₂ is independently alkylene of 1 to about 20 carbons. More preferably, each R₂ is independently alkylene of 1 to about 10 carbons. Even more 5 preferably, each R₂ is independently alkylene of 1 to about 5 carbons, with methylene being especially preferred. In alternate preferred embodiments, R₂ is a direct bond.

In the above formula, each of R₃ and R₄ is independently hydrogen or alkyl which ranges from 1 to about 10 carbons, and all combinations and subcombinations of ranges therein. Preferably, each of R₃ and R₄ is hydrogen or alkyl of 1 to about 5 carbons. 10 More preferably, each of R₃ and R₄ is hydrogen.

In the above formula, each R₅ is independently a direct bond or alkylene which ranges from 1 to about 30 carbons, and all combinations and subcombinations of ranges therein. Preferably, each R₅ is independently a direct bond or alkylene of 1 to about 20 carbons. More preferably, each R₅ is independently a direct bond or alkylene of 1 to 15 about 10 carbons. Even more preferably, each R₅ is independently a direct bond or alkylene of 1 to about 5 carbons. Still more preferably, each R₅ is a direct bond or -(CH₂)_y-, where x is 1 or 2.

In the above compound, when three or more of X₁, X₂, X₃, R₂ and M are direct bonds, then Z is a hydrophilic polymer. Preferably, when two or more of X₁, X₂, X₃, 20 R₂ and M are direct bonds, then Z is a hydrophilic polymer. More preferably, when one or more of X₁, X₂, X₃, R₂ and M are direct bonds, then Z is a hydrophilic polymer.

Also in the above compound, when Z is a direct bond, then three or more of X₁, X₂, X₃, R₂ and M are other than direct bonds. Preferably, when Z is a direct bond, four or more of X₁, X₂, X₃, R₂ and M are other than direct bonds. More preferably, when Z is a 25 direct bond, all of X₁, X₂, X₃, R₂ and M are other than direct bonds.

There are also provided herein compounds of the formula

L-P-T

wherein:

L is a hydrophobic compound;

30 P is a linking group; and

T is a targeting ligand.

Generally speaking, L may be selected from any of a variety of suitable hydrophobic compounds, such as a biocompatible lipid, including the lipids discussed above. In preferred embodiments, L is a lipid selected from the group consisting of lecithins, phosphatidylcholines, phosphatidylserines, phosphatidylinositols, cardiolipins, 5 cholesterolamines, lysophosphatides, erythro-sphingosines, sphingomyelins, ceramides, cerebrosides, saturated phospholipids, unsaturated phospholipids, krill phospholipids, fatty acids and steroids. More preferably, L is a lipid selected from the group consisting of lecithins, phosphatidylcholines, phosphatidylserines, phosphatidylinositols, fatty acids and steroids. Even more preferably, L is a lipid selected from the group consisting of 10 1,2-diacyl-sn-glycero-3-phosphocholines, 1,2-diacyl-sn-glycero-3-phosphoethanolamines, 1,2-diacyl-sn-glycero-3-[phospho-rac-(1-glycerols)], 1,2-diacyl-sn-glycero-3-phosphates, 1,2-diacyl-sn-glycero-3-[phospho-serines], lysophosphatidylcholines, lysophosphatidylglycerols, 1,2-diacyl-sn-glycerols, 1,2-diacylethylene glycols such as, for example, such as 1,2-dioctanoyl ethylene glycol, 1,2-dicaproyl ethylene glycol, 1,2- 15 dilauroyl ethylene glycol, 1,2-dimyristoyl ethylene glycol, 1,2-dipalmitoyl ethylene glycol and 1,2-dioleoyl ethylene glycol, N-(n-caproylamine)-1,2-diacyl-sn-glycero-3-phosphoethanolamines, N-dodecanylamine-1,2-diacyl-sn-glycero-3-phosphoethanolamines, Nsuccinyl-1,2-diacyl-sn-glycero-3-phosphoethanolamines, N-glutaryl-1,2-diacyl-sn-glycero-3-phosphoethanolamines, N-dodecanyl-1,2-diacyl-sn-glycero-3-phosphoethanolamines, fatty acids, such as stearic acid, and steroids, such as cholesterol.

In other preferred embodiments, L is a protein which comprises albumin. In still other preferred embodiments, L is a polymer which comprises synthetic polymers or copolymers prepared from monomers selected from the group consisting of acrylic acid, methacrylic acid, ethyleneimine, crotonic acid, acrylamide, ethyl acrylate, methyl 25 methacrylate, 2-hydroxyethyl methacrylate, lactic acid, glycolic acid, ϵ -caprolactone, acrolein, cyanoacrylate, bisphenol A, epichlorhydrin, hydroxyalkylacrylates, siloxane, dimethylsiloxane, ethylene oxide, propylene oxide, ethylene glycol, hydroxyalkylmethacrylates, N-substituted acrylamides, N-substituted methacrylamides, N-vinyl-2-pyrrolidone, 2,4-pentadiene-1-ol, vinyl acetate, acrylonitrile, styrene, p-amino-styrene, 30 p-aminobenzylstyrene, sodium styrene sulfonate, sodium 2-sulfoxyethyl-methacrylate, vinyl pyridine, aminoethyl methacrylates and 2-methacryloyloxytrimethyl-ammonium

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chloride. Also preferred are compounds where L is a polymer which comprises synthetic polymers or copolymers selected from the group consisting of polyacrylic acid, polyethyleneimine, polymethacrylic acid, polymethylmethacrylate, polysiloxane, polydimethylsiloxane, polylactic acid, poly(ϵ -caprolactone), epoxy resin, poly(ethylene oxide), poly(propylene oxide), poly(ethylene glycol), polyamide, polyvinylidene-polyacrylonitrile, polyvinylidene-polyacrylonitrile-polymethylmethacrylate and polystyrene-polyacrylonitrile. Preferred among these polymers is polyvinylidene-polyacrylonitrile copolymer.

In the above compounds, P is a linking group of variable length. In 10 preferred embodiments, P is a hydrophilic polymer. In more preferred embodiments, P is a hydrophilic polymer selected from the group consisting of polyalkyleneoxides, polyvinyl alcohol, polyvinylpyrrolidones, polyacrylamides, polymethacrylamides, polyphosphazenes, poly(hydroxyalkylcarboxylic acids) and polyoxazolidines. More preferably, P is a polyalkyleneoxide polymer, with polyethylene glycol and polypropylene 15 glycol being even more preferred and polyethylene glycol being particularly preferred.

In the above formula, T is a targeting ligand. In preferred embodiments, T is selected from among the various targeting ligands discussed in detail above. Thus, for example, T preferably targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein 20 GPIIbIIIa receptor. In certain preferred embodiments, T targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells and the glycoprotein GPIIbIIIa receptor. In addition, T is preferably selected from the group consisting of proteins, peptides, saccharides, steroids, steroid analogs, bioactive agents, and genetic material. In these latter embodiments, T is preferably selected from the group 25 consisting of proteins, peptides and saccharides. In embodiments where T comprises a peptide, T preferably comprises the sequences Arg-Gly-Asp (RGD) or Ala-Gly-Asp (AGD), such as Lys-Gln-Ala-Gly-Asp-Val (KQAGDV), or T is a cyclic peptide, such as DMP 728. (See, e.g., Mousa et al., *Thrombosis Research*, Vol. 76 (2), pp. 109-119 (1994), the disclosures of which are hereby incorporated herein by reference, in their entirety.) 30 Also preferred are targeting ligands which target regions of arteriosclerosis, especially atherosclerotic plaque.

- In the case of targeting ligands which comprise saccharide groups, suitable saccharide moieties include, for example, monosaccharides, disaccharides and polysaccharides. Exemplary monosaccharides may have six carbon atoms and these saccharides include allose, altrose, glucose, mannose, gulose, idose, galactose, talose,
- 5 fructose, psicose, verbose and tagatose. Five carbon saccharids include ribose, arabinose, xylose, lyxose, ribulose and xylulose. Four carbon saccharides include erythrose, threose and erythrulose. Disaccharides include sucrose, lactose, maltose, isomaltose and cellobiose. Saccharide bearing targeting lipids may be synthesized through a multistep organic synthesis approach, as described more fully hereinafter. For example, lipids
- 10 bearing targeting glucose moieties may be prepared by reacting, for example, α -glucopyranosyl bromide tetrabenzyl with ω -trifluoroacetylaminopolyethyleneglycol to obtain ω -glucopyranosyl tetrabenzyl- ω' -trifluoroacetylaminopolyethyleneglycol. This may then be hydrolyzed in a sodium carbonate or potassium carbonate solution and then hydrogenated to obtain ω -glucopyranosyl- ω' -amino-polyethyleneglycol.
- 15 Aminoglycopyranosyl terminated polyethyleneglycol may then react with N-DPGS-succinimide to form the lipid bearing saccharide DPGS-NH-PEG-Glucose. Also preferred are targeting ligands which target infarcted myocardium. In certain embodiments, the targeting ligands target cancer cells.

The foregoing preferred embodiments of the compounds of the present invention are preferred for various reasons, including ease of synthesis, diagnostic efficacy, enhanced biocompatibility, and/or improved targeting efficacy.

The targeting ligand may be incorporated in the present compositions in a variety of ways. Generally speaking, the targeting ligand may be incorporated in the present compositions by being associated covalently or non-covalently with one or more of the materials which are included in the compositions, including, for example, the lipids, proteins, polymers and/or auxiliary stabilizing materials. In preferred form, the targeting ligand is associated covalently with one or more of the aforementioned materials contained in the present compositions. As noted above, preferred compositions of the present invention comprise lipid, protein or polymer compounds. In these compositions, the targeting ligands are preferably associated covalently with the lipid, protein or polymer compounds.

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Exemplary covalent bonds by which the targeting ligands are associated with the lipids, proteins, polymers and/or vesicles include, for example, amide (-CONH-); thioamide (-CSNH-); ether (ROR', where R and R' may be the same or different and are other than hydrogen); ester (-COO-); thioester (-COS-); -O-; -S-; -S_n-, where n is greater than 1, preferably about 2 to about 8, and more preferably about 2; carbamates; -NH-; -NR-, where R is alkyl, for example, alkyl of from 1 to about 4 carbons; urethane; and substituted imidate; and combinations of two or more of these. Covalent bonds between targeting ligands and, for example, lipids, may be achieved through the use of molecules that may act as spacers to increase the conformational and topographical flexibility of the ligand. Examples of such spacers include, for example, succinic acid, 1,6-hexanedioic acid, 1,8-octanedioic acid, and the like, as well as modified amino acids, such as, for example, 6-aminohexanoic acid, 4-aminobutanoic acid, and the like. In addition, in the case of targeting ligands which comprise peptide moieties, sidechain-to-sidechain crosslinking may be complemented with sidechain-to-end crosslinking and/or end-to-end crosslinking. Also, small spacer molecules, such as dimethylsuberimidate, may be used to accomplish similar objectives. The use of agents, including those used in Schiff's base-type reactions, such as gluteraldehyde, may also be employed. The Schiff's base linkages, which may be reversible linkages, can be rendered more permanent covalent linkages via the use of reductive amination procedures. This may involve, for example, chemical reducing agents, such as lithium aluminum hydride reducing agents or their milder analogs, including lithium aluminum diisobutyl hydride (DIBAL), sodium borohydride (NaBH₄) or sodium cyanoborohydride (NaBH₃CN).

The covalent linking of the targeting ligands to the materials in the present compositions, including the lipids, proteins and/or polymers, may be accomplished using synthetic organic techniques which would be readily apparent to one of ordinary skill in the art, based on the present disclosure. For example, the targeting ligands may be linked to the materials, including the lipids, via the use of well known coupling or activation agents. As known to the skilled artisan, activating agents are generally electrophilic. This electrophilicity can be employed to elicit the formation of a covalent bond. Exemplary activating agents which may be used include, for example, carbonyldiimidazole (CDI),

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dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), methyl sulfonyl chloride, Castro's Reagent, and diphenyl phosphoryl chloride.

The covalent bonds may involve crosslinking and/or polymerization.

Crosslinking preferably refers to the attachment of two chains of polymer molecules by 5 bridges, composed of either an element, a group, or a compound, which join certain carbon atoms of the chains by covalent chemical bonds. For example, crosslinking may occur in polypeptides which are joined by the disulfide bonds of the cystine residue. Crosslinking may be achieved, for example, by (1) adding a chemical substance (cross-linking agent) and exposing the mixture to heat, or (2) subjecting a polymer to high energy radiation. A 10 variety of crosslinking agents, or "tethers", of different lengths and/or functionalities are described, for example, in R.L. Lunblad, *Techniques in Protein Modification*, CRC Press, Inc., Ann Arbor, MI, pp. 249-68 (1995), the disclosures of which are hereby incorporated herein by reference, in their entirety. Exemplary crosslinkers include, for example, 3,3'-dithiobis(succinimidylpropionate), dimethyl suberimidate, and its variations thereof, based 15 on hydrocarbon length, and bis-N-maleimido-1,8-octane.

In accordance with preferred embodiments, the targeting ligands may be linked or attached to the lipids, proteins or polymers, or other stabilizing materials, via a linking group. A variety of linking groups are available and would be apparent to one skilled in the art, once armed with the present disclosure. Preferably, the linking group 20 comprises a hydrophilic polymer. Suitable hydrophilic linker polymers include, for example, polyalkyleneoxides such as, for example, polyethylene glycol (PEG) and polypropylene glycol (PPG), polyvinylpyrrolidones, polyvinylmethylethers, polyacrylamides, such as, for example, polymethacrylamides, polydimethylacrylamides and polyhydroxypropylmethacrylamides, polyhydroxyethyl acrylates, polyhydroxypropyl 25 methacrylates, polymethyloxazolines, polyethyloxazolines, polyhydroxyethyloxazolines, polyhydroxypropyloxazolines, polyvinyl alcohols, polyphosphazenes, poly(hydroxyalkylcarboxylic acids), polyoxazolidines, and polyaspartamide. The hydrophilic polymers are preferably selected from the group consisting of PEG, PPG, polyvinylalcohol and polyvinylpyrrolidone and copolymers thereof, with PEG and PPG 30 polymers being more preferred and PEG polymers being even more preferred. Thus, in embodiments involving lipid compositions which comprise lipids bearing polymers

including, for example, DPPE-PEG, the targeting ligand may be linked directly to the polymer which is attached to the lipid to provide, for example, a conjugate of DPPE-PEG-TL, where TL is a targeting ligand. Thus, using the example DPPE-PEG, such as, for example, DPPE-PEG5000, the aforementioned conjugate may be represented as DPPE-
5 PEG5000-TL. The hydrophilic polymer used as a linking group is preferably a bifunctional polymer, for example, bifunctional PEG, such as diamino-PEG. In this case, one end of the PEG group is linked, for example, to a lipid compound, and is bound at the free end to the targeting ligand via an amide linkage. A hydrophilic polymer, for example, PEG, substituted with a terminal carboxylate group on one end and a terminal amino group
10 on the other end, may also be used. These latter bifunctional hydrophilic polymer may be preferred since they possess various similarities to amino acids.

Standard peptide methodology may be used to link the targeting ligand to the lipid when utilizing linker groups having two unique terminal functional groups.

Bifunctional hydrophilic polymers, and especially bifunctional PEGs, may be synthesized
15 using standard organic synthetic methodologies. In addition, many of these materials are available commercially. For example, α -amino, ω -carboxy PEG is commercially available from Shearwater Polymers (Huntsville, AL). An advantage of using a PEG material as the linking group is that the size of the PEG can be varied such that the number of monomeric subunits of ethylene glycol may be as few as, for example, about 5, or as many as, for
20 example, about 500 or even greater. Accordingly, the "tether" or length of the linkage may be varied, as desired. This may be important depending, for example, on the particular targeting ligand employed. For example, a targeting ligand which comprises a large protein molecule may require a short tether, such that it will simulate a membrane bound protein. A short tether would also allow for a vesicle to maintain a close proximity to the
25 cell. This can be used advantageously in connection with vesicles which also comprise a bioactive agent, in that the concentration of bioactive agent which is delivered to the cell may be advantageously increased.

Another suitable linking group which may provide a short tether is glyceraldehyde. Glyceraldehyde may be bound, for example, to DPPE via a Schiff's base
30 reaction. Subsequent Amadori rearrangement can provide a substantially short linking

group. The β carbonyl of the Schiff's base may then react with a lysine or arginine of the targeting protein or peptide to form the targeted lipid.

More specifically, the compounds employed in the present compositions, including lipids, proteins and/or polymers, may contain various functional groups, such as,

5 for example, hydroxy, thio and amine groups, which can react with a carboxylic acid or carboxylic acid derivative of the hydrophilic polymeric linker using suitable coupling conditions which would be apparent to one of ordinary skill in the art, once armed with the present disclosure. After the carboxylic acid group (or derivative thereof) reacts with the functional group, for example, hydroxy, thio or amine group to form an ester, thioester or

10 amide group, any protected functional group may be deprotected utilizing procedures which would be well known to those skilled in the art. The term protecting group, as used herein, refers to any moiety which may be used to block reaction of a functional group and which may be removed, as desired, to afford the unprotected functional group. Any of a variety of protecting groups may be employed and these will vary depending, for example,

15 as to whether the group to be protected is an amine, hydroxyl or carboxyl moiety. If the functional group is a hydroxyl group, suitable protecting groups include, for example, certain ethers, esters and carbonates. Such protecting groups are described, for example, in Greene, TW and Wuts, PGM "Protective Groups in Organic Synthesis" John Wiley, New York, 2nd Edition (1991), the disclosures of which are hereby incorporated herein by reference, in their entirety. Exemplary protecting groups for amine groups include, for

20 example, t-butyloxycarbonyl (Boc), benzyloxycarbonyl(Cbz), o-nitrobenzyloxycarbonyl and trifluoroacetate (TFA).

Amine groups which may be present, for example, on a backbone of a polymer which is included in the vesicles, may be coupled to amine groups on a

25 hydrophilic linking polymer by forming a Schiff's base, for example, by using coupling agents, such as glutaraldehyde. An example of this coupling is described by Allcock et al., *Macromolecules* Vol. 19(6), pp. 1502-1508 (1986), the disclosures of which are hereby incorporated herein by reference, in their entirety. If, for example, vesicles are formulated from polylysine, free amino groups may be exposed on the surface of the

30 vesicles, and these free amine groups may be activated as described above. The activated amine groups can be used, in turn, to couple to a functionalized hydrophilic polymer, such

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as, for example, α -amino- ω -hydroxy-PEG in which the ω -hydroxy group has been protected with a carbonate group. After the reaction is completed, the carbonate group can be cleaved, thereby enabling the terminal hydroxy group to be activated for reaction to a suitable targeting ligand. In certain embodiments, the surface of a vesicle may be
5 activated, for example, by displacing chlorine atoms in chlorine-containing phosphazene residues, such as polydichlorophosphazine. Subsequent addition of a targeting ligand and quenching of the remaining chloride groups with water or aqueous methanol will yield the coupled product.

In addition, poly(diphenoxypyrophosphazene) can be synthesized (Allcock et
10 al., *Macromolecules* Vol. (1986) 19(6), pp. 1502-1508) and immobilized, for example, on DPPE, followed by nitration of the phenoxy moieties by the addition of a mixture of nitric acid and acetic anhydride. The subsequent nitro groups may then be activated, for example, by (1) treatment with cyanogen bromide in 0.1 M phosphate buffer (pH 11), followed by addition of a targeting ligand containing a free amino moiety to generate a
15 coupled urea analog, (2) formation of a diazonium salt using sodium nitrite/HCl, followed by addition of the targeting ligand to form a coupled ligand, and/or (3) the use of a dialdehyde, for example, glutaraldehyde as described above, to form a Schiff's base. After linking the DPPE to the hydrophilic polymer and the targeting ligand, the vesicles may be formulated utilizing the procedures described herein.

20 Aldehyde groups on polymers can be coupled with amines as described above by forming a Schiff's base. An example of this coupling procedure is described in Allcock and Austin *Macromolecules* vol 14. p1616 (1981), the disclosures of which are hereby incorporated herein by reference, in their entirety.

In the above procedures, the polymer or terminus of the lipid, for example,
25 phosphatidylglycerol or phosphatidylethanolamine, is preferably activated and coupled to the hydrophilic polymeric linker, the terminus of which has been blocked in a suitable manner. As an example of this strategy, α -amino, ω -carboxy PEG-4000 having a t-Boc protected terminal amino group and a free carboxylate end, may be activated with 1,1'-carbonyldiimidazole in the presence of hydroxybenzotriazole in N-methylpyrrolidone.
30 After the addition of phosphatidylethanolamine, the t-Boc group may be removed by using trifluoroacetic acid (TFA), leaving the free amine. The amine may then be reacted with a

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- targeting ligand which may comprise, for example, a peptide, protein, alkaloid, or other moiety, by similar activation of the ligand, to provide the lipid-linker-targeting ligand conjugate. Other strategies, in addition to those exemplified above, may be utilized to prepare the lipid-linker-targeting ligand conjugates. Generally speaking, these methods
- 5 employ synthetic strategies which are generally known to those skilled in the art of synthetic organic chemistry.

As known to one of ordinary skill in the art, immunoglobulins typically comprise a flexible region which is identified as the "hinge" region. *See, e.g., "Concise Encyclopedia of Biochemistry", Second Edition, Walter de Gruyter & Co., pp. 282-283*

10 (1988). Fab' fragments can be linked to the lipids, polymers, proteins and/or vesicles using the well-defined sites of the thiols of the hinge region. This is a preferred region for coupling Fab' fragments as the potential binding site is remote from the antigen-recognition site. Generally speaking, it may be difficult to utilize the thiols of the hinge group unless they are adequately prepared. In particular, as outlined by Shahinian and

15 Salvias (*Biochimica et Biophysica Acta*, Vol 1239 (1995) 157-167) it may be important to reduce the thiol groups so that they are available for coupling, for example, to maleimide derivatized linking groups. Examples of reducing agents commonly used are ethanedithiol, mercaptoethanol, mercaptoethylamine or the more commonly used dithiothreitol, commonly referred to as Cleland's reagent. However, it should be noted

20 that care should be exercised when utilizing certain reducing agents, such as dithiothreitol, as overreduction may result. Discriminating use of reducing agents may be necessary in connection with proteins whose activity or binding capacity may be compromised due to overreduction and subsequent denaturation or conformational change. *See, e.g., Shahinian, S. and Salvias, J.R. (1995) Biochim. Biophys. Acta 1239, 157 - 167.*

25 F(ab')₂ antibody fragments may be prepared by incubating the antibodies with pepsin (60μg/ml) in 0.1 M sodium acetate (pH 4.2) for 4 h at 37°C. Digestion may be terminated by adding 2 M Tris (pH 8.8) to a final concentration of 80 mM. The F(ab')₂ fragments may then be obtained by centrifugation (10,000 x g. 30 min. 4°C). The supernatant may then be dialyzed at 4°C against 150 mM NaCl, 20 mM phosphate at pH

30 7.0. This then may be chromatographed on a column of Protein A-Sepharose CL-4B to remove any undigested IgG. The Fab' fragments may then be prepared by extensively

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degassing the solutions and purging with nitrogen prior to use. The F(ab')₂ fragments may be provided at a concentration of 5 mg/ml and reduced under argon in 30 mM cysteine. Alternatively, cysteamine may be employed. 100 mM Tris, pH 7.6 may be used as a buffer for 15 min at 37°C. The solutions may then be diluted 2-fold with an equal volume
5 of the appropriate experimental buffer and spun through a 0.4 ml spin column of Bio-Gel P-6DG. The resulting Fab' fragments may be more efficient in their coupling to maleimide linkers. Note also that the same procedure may be employed with other macromolecules containing cysteine residues for coupling, for example, to the maleimide spacers. Also, peptides may be utilized provided that they contain a cysteine residue. If
10 the peptides have not been made fresh and there is a possibility of oxidation of cysteine residues within the peptide structure, it may be necessary to regenerate the thiol group using the approach outlined above.

Additional linkers would include other derivatives of lipids useful for coupling to a bifunctional spacer. For example, phosphatidylethanolamine (PE) may be
15 coupled to a bifunctional agent. For example N-succinimidyl 4-(p-maleimido-phenyl)butyrate (SMPB) and N-succinimidyl 3-(2-pyridyldithiol) propionate (SPDP), N-succinimidyl trans-4-(N-maleimidylmethyl)cyclohexane-1-carboxylate (SMCC), and N-succinimidyl 3-maleimidylbenzoate (SMB) may be used among others, to produce, for example the functionalized lipids MPB-PE and PDP-PE.

20 The free end of the hydrophilic spacer, such as polyethylene glycol ethylamine, which contains a reactive group, such as an amine or hydroxyl group, could be used to bind a cofactor or other targeting ligand. For example, polyethylene glycol ethylamine may be reacted with N-succinimidylbiotin or p-nitrophenylbiotin to introduce onto the spacer a useful coupling group. For example, biotin may be coupled to the spacer
25 and this will readily bind non-covalently proteins. As an example, MPB-PEG-DPPE may be synthesized as follows. DPPE-PEG with a free amino group at the terminus of the PEG will be provided as described previously.. Synthesis of the SMPB:PEG-DPPE may then be carried out with 1 equivalent of triethylamine in chloroform at a molar ratio of 1:5 SMPB:DPPE-PEG. After 3 hours, the reaction mixture will be evaporated to dryness
30 under argon. Excess unreacted SMPB and major by products will be removed by preparative thin layer chromatography (TLC, silica gel developed with 50% acetone in

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- chloroform). The upper portion of the lipid band can be extracted from the silica with about 20-30% methanol in chloroform (V:V) resulting in the isolation of pure intact MPB-Peg-DPPE. Streptavidin may then be coupled to proteins so that the proteins in turn may then be coupled to the MPB-PEG-DPPE. Briefly SPDP would be incubated with
- 5 streptavidin at room temperature for 30 minutes and chromatography employed to remove unreacted SPDP. Dithiothreitol (DTT) was added to the reaction mixture and 10 minutes later 2-thiopyridone at a concentration of 343 nM. The remainder of the reaction mixture is reduced with DTT (25 mM for 10 min.). The thiolated product is isolated by gel exclusion. The resulting streptavidin labeled proteins may then be used to bind to the
- 10 biotinylated spacers affixed to the lipid moieties.

Additional methods which may be employed for covalently linking targeting ligands to the lipids, proteins or polymers, vesicles, or other stabilizing materials, are described, for example, in copending U.S. application Serial No. 08/851,780, filed May 6, 1997 and copending U.S. application Serial No. 08/887,215, filed July 2, 1997, the

15 disclosures of which are incorporated herein by reference, in their entireties.

In preferred embodiments of the present invention, the targeted compounds, namely, targeted lipids, proteins, and polymers, may be incorporated in compositions which are used to form targeted vesicles, including, for example, targeted micelles, targeted liposomes, targeted albumin coated microspheres, and/or targeted polymer coated

20 microspheres. The targeting ligand which is attached to the compounds from which the vesicles are prepared may be directed, for example, outwardly from the surface of the vesicle. Thus, there is provided a targeted vesicle which can be used to target receptors and tissues.

In certain embodiments, the targeting ligands may be incorporated in the

25 present compositions via non-covalent associations. As known to those skilled in the art, non-covalent association is generally a function of a variety of factors, including, for example, the polarity of the involved molecules, the charge (positive or negative), if any, of the involved molecules, the extent of hydrogen bonding through the molecular network, and the like. Non-covalent bonds are preferably selected from the group consisting of

30 ionic interaction, dipole-dipole interaction, hydrogen bonds, hydrophilic interactions, van der Waal's forces, and any combinations thereof. Non-covalent interactions may be

employed to bind the targeting ligand to the lipid, or directly to the surface of a vesicle. For example, the amino acid sequence Gly-Gly-His may be bound to the surface of a vesicle, preferably by a linker, such as PEG, and copper, iron or vanadyl ion may then be added. Proteins, such as antibodies which contain histidine residues, may then bind to the 5 vesicle via an ionic bridge with the copper ion, as described in U.S. Patent No. 5,466,467, the disclosures of which are hereby incorporated herein by reference, in their entirety. An example of hydrogen bonding involves cardiolipin lipids which can be incorporated into the lipid compositions.

In preferred embodiments of the present invention, which may involve 10 vesicle compositions, changes, for example, in pH and/or temperature *in vivo*, may be employed to promote a change in location in the targeting ligands, for example, from a location within the vesicle, to a location external to the outer wall of the vesicle. This may promote binding of the targeting ligands to targeting sites, for example, receptors, such as the GPIIbIIIa receptor, and tissues, including myocardial, endothelial and epithelial cells, 15 since the targeting ligand has a greater likelihood of exposure to such targeting sites. In addition, high energy ultrasound can be used to promote rupturing of the vesicles. This can also expose the targeting ligand to the desired binding site.

The concentration of targeting ligand employed in the present compositions may vary depending, for example, on the particular targeting agent employed, the receptor 20 or tissue being targeted, the other components of the compositions, whether the targeting ligand is associated covalently and/or non-covalently with other components of the compositions, for example, lipids, proteins, polymers or vesicles and the like. Typically, the concentration of targeting ligand in the present compositions may be initiated at lower levels and increased until the desired contrast enhancement effect is achieved. Targeted 25 hydrophobic compounds (i.e., for example, conjugates in which the targeting ligand is linked covalently to, for example, a lipid, as in the case of compounds of the formula L-P-T), as well as unbound or free targeting ligand (as in, for example, embodiments in which the targeting ligand may be associated non-covalently with a stabilizing compound, such as a lipid), may be employed in the compositions in a concentration which ranges 30 from about 0.05 wt % to about 10 wt % (and all combinations and subcombinations of ranges therein), based on the weight of unbound stabilizing materials employed in the

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- composition. Preferably, the conjugate or free targeting ligand may be employed in the compositions in a concentration from about 0.5 wt % to about 5 wt %, with concentrations of from about 1 wt % to about 3 wt % being more preferred. Even more preferably, the conjugate or free targeting ligand may be employed in the present compositions in a
- 5 concentration of from about 1 wt % to about 2 wt %, with a concentration of about 1.25 wt % being especially preferred.

As would be apparent to the skilled artisan, once armed with the teachings of the present invention, the concentration of targeted hydrophobic compounds and/or unbound or free targeting ligand that may be employed in the compositions of the present

10 invention may also be expressed in mole %. In this connection, targeted hydrophobic compounds and/or unbound or free targeting ligand may be employed in the compositions of the present invention in a concentration which ranges from 0.05 mole % to about 10 mole % (and all combinations and subcombinations of ranges therein), based on the number of moles of unbound stabilizing materials employed in the composition.

15 Preferably, the conjugate or free targeting ligand may be employed in the compositions in a concentration from about 0.5 mole % to about 5 mole %, with concentrations of from about 1 mole % to about 3 mole % being more preferred. Even more preferably, the conjugate or free targeting ligand may be employed in the present compositions in a concentration of from about 1 mole % to about 2 mole %, with a concentration of about

20 1.8 mole % being especially preferred.

As noted above, the present lipid and/or vesicle compositions are desirably formulated in an aqueous environment. This can induce the lipid, because of its hydrophobic/hydrophilic nature, to form vesicles, which may be the most stable configuration which can be achieved in such an environment. The diluents which can be

25 employed to create such an aqueous environment include, for example, water, including deionized water or water containing one or more dissolved solutes, such as salts, which preferably do not interfere with the formation and/or stability of the vesicles or their use as diagnostic agents, such as ultrasound contrast agents, MRI contrast agents or CT contrast agents; and normal saline and physiological saline.

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The lipid and/or vesicle compositions of the present invention may also comprise additional contrast agents, including conventional contrast agents, which may serve to increase their effectiveness as contrast agents for diagnostic imaging.

- Accordingly, it is within the scope of the present invention to provide
- 5 vesicle compositions which comprise flexible vesicles, for example, vesicles formulated from lipids, or inflexible vesicles, for example, vesicles prepared from polymethyl methacrylate.

It is contemplated that the compositions of the present invention are particularly useful in connection with ultrasound, including diagnostic and therapeutic

10 ultrasound. The use of the present compositions in ultrasound is described throughout the present disclosure.

As noted above, the present compositions may also be employed in connection with computed tomography (CT) imaging. CT suffers from various drawbacks, and is generally less effective as compared to the diagnostic techniques

15 discussed above. Nevertheless, if a high enough concentration of the present contrast media, and especially gas filled vesicle compositions, is delivered to the region of interest, for example, a blood clot, the clot can be detected on the CT images by virtue of a decrease in the overall density of the clot. In general, a concentration of about 1/10 of 1% of gas filled vesicles or higher (on a volume basis), may be needed to delivered to the

20 region of interest, including the aforementioned blood clot, to be detected by CT.

Exemplary paramagnetic contrast agents suitable for use in the present compositions include, for example, stable free radicals, such as, for example, stable nitroxides, as well as compounds comprising transition, lanthanide and actinide elements, which may, if desired, be in the form of a salt or may be covalently or non-covalently

25 bound to complexing agents, including lipophilic derivatives thereof, or to proteinaceous macromolecules.

Preferable transition, lanthanide and actinide elements include, for example, Gd(III), Mn(II), Cu(II), Cr(III), Fe(II), Fe(III), Co(II), Er(II), Ni(II), Eu(III) and Dy(III). More preferably, the elements may be Gd(III), Mn(II), Cu(II), Fe(II), Fe(III), Eu(III) and

30 Dy(III), especially Mn(II) and Gd(III).

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The foregoing elements may, if desired, be in the form of a salt, including inorganic salts, such as a manganese salt, for example, manganese chloride, manganese carbonate, manganese acetate, and organic salts, such as manganese gluconate and manganese hydroxylapatite. Other exemplary salts include salts of iron, for example, iron sulfides and ferric salts such as ferric chloride.

- These elements may also, if desired, be bound, for example, through covalent or noncovalent association, to complexing agents, including lipophilic derivatives thereof, or to proteinaceous macromolecules. Preferable complexing agents include, for example, diethylenetriaminepentaacetic acid (DTPA), ethylene-diaminetetraacetic acid (EDTA), 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA), 1,4,7,10-tetraazacyclododecane-N,N',N''-triacetic acid (DOTA), 3,6,9-triaza-12-oxa-3,6,9-tricarboxymethylene-10-carboxy-13-phenyl-tridecanoic acid (B-19036), hydroxybenzylethylenediamine diacetic acid (HBED), N,N'-bis(pyridoxyl-5-phosphate)ethylene diamine, N,N'-diacetate (DPDP), 1,4,7-triazacyclononane-N,N',N''-triacetic acid (NOTA), 1,4,8,11-tetraazacyclotetradecane-N,N',N'',N'''-tetraacetic acid (TETA), kryptands (macrocyclic complexes), and desferrioxamine. More preferably, the complexing agents are EDTA, DTPA, DOTA, DO3A and kryptands, most preferably DTPA. Preferable lipophilic complexes include alkylated derivatives of the complexing agents EDTA, DOTA, for example, N,N'-bis-(carboxydecylamidomethyl-N-2,3-dihydroxypropyl)-ethylenediamine-N,N'-diacetate (EDTA-DDP); N,N'-bis-(carboxy-octadecylamido-methyl-N-2,3-dihydroxypropyl)ethylenediamine-N,N'-diacetate (EDTA-ODP); N,N'-Bis(carboxy-laurylamidomethyl-N-2,3-dihydroxypropyl)ethylenediamine-N,N'-diacetate (EDTA-LDP); and the like, including those described in U.S. Patent No. 5,312,617, the disclosures of which are hereby incorporated herein by reference, in their entirety. Preferable proteinaceous macromolecules include, for example, albumin, collagen, polyarginine, polylysine, polyhistidine, γ -globulin and β -globulin, with albumin, polyarginine, polylysine, and polyhistidine being more preferred.

Suitable complexes therefore include Mn(II)-DTPA, Mn(II)-EDTA, Mn(II)-DOTA, Mn(II)-DO3A, Mn(II)-kryptands, Gd(III)-DTPA, Gd(III)-DOTA, Gd(III)-DO3A, Gd(III)-kryptands, Cr(III)-EDTA, Cu(II)-EDTA, or iron-desferrioxamine, especially Mn(II)-DTPA or Gd(III)-DTPA.

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Nitroxides are paramagnetic contrast agents which increase both T1 and T2 relaxation rates on MRI by virtue of the presence of an unpaired electron in the nitroxide molecule. As known to one of ordinary skill in the art, the paramagnetic effectiveness of a given compound as an MRI contrast agent may be related, at least in part, to the number of 5 unpaired electrons in the paramagnetic nucleus or molecule, and specifically, to the square of the number of unpaired electrons. For example, gadolinium has seven unpaired electrons whereas a nitroxide molecule has one unpaired electron. Thus, gadolinium is generally a much stronger MRI contrast agent than a nitroxide. However, effective correlation time, another important parameter for assessing the effectiveness of contrast 10 agents, confers potential increased relaxivity to the nitroxides. When the tumbling rate is slowed, for example, by attaching the paramagnetic contrast agent to a large molecule, it will tumble more slowly and thereby more effectively transfer energy to hasten relaxation of the water protons. In gadolinium, however, the electron spin relaxation time is rapid and will limit the extent to which slow rotational correlation times can increase relaxivity.

15 For nitroxides, however, the electron spin correlation times are more favorable and tremendous increases in relaxivity may be attained by slowing the rotational correlation time of these molecules. The gas filled vesicles of the present invention are ideal for attaining the goals of slowed rotational correlation times and resultant improvement in relaxivity. Although not intending to be bound by any particular theory of operation, it is 20 contemplated that since the nitroxides may be designed to coat the perimeters of the vesicles, for example, by making alkyl derivatives thereto, the resulting correlation times can be optimized. Moreover, the resulting contrast medium of the present invention may be viewed as a magnetic sphere, a geometric configuration which maximizes relaxivity.

If desired, the nitroxides may be alkylated or otherwise derivatized, such as 25 the nitroxides 2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical, and 2,2,6,6-tetramethyl-1-piperidinyloxy, free radical (TMPO).

Exemplary superparamagnetic contrast agents suitable for use in the compositions of the present invention include metal oxides and sulfides which experience a magnetic domain, ferro- or ferrimagnetic compounds, such as pure iron, magnetic iron 30 oxide, such as magnetite, γ -Fe₂O₃, Fe₃O₄, manganese ferrite, cobalt ferrite and nickel ferrite. Paramagnetic gases can also be employed in the present compositions, such as

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oxygen 17 gas ($^{17}\text{O}_2$). In addition, hyperpolarized xenon, neon, or helium gas may also be employed. MR whole body imaging may then be employed to rapidly screen the body, for example, for thrombosis, and ultrasound may be applied, if desired, to aid in thrombolysis.

- The contrast agents, such as the paramagnetic and superparamagnetic contrast agents described above, may be employed as a component within the lipid and/or vesicle compositions. In the case of vesicle compositions, the aforementioned contrast agents may be entrapped within the internal void thereof, administered as a solution with the vesicles, incorporated with any additional stabilizing materials, or coated onto the surface or membrane of the vesicle.
- If desired, the paramagnetic or superparamagnetic agents may be delivered as alkylated or other derivatives incorporated into the compositions, especially the lipidic walls of the vesicles. In particular, the nitroxides 2,2,5,5-tetramethyl-1-pyrrolidinyloxy, free radical and 2,2,6,6-tetramethyl-1-piperidinyloxy, free radical, can form adducts with long chain fatty acids at the positions of the ring which are not occupied by the methyl groups via a variety of linkages, including, for example, an acetoxy linkage. Such adducts are very amenable to incorporation into the lipid and/or vesicle compositions of the present invention.

Mixtures of any one or more of the paramagnetic agents and/or superparamagnetic agents in the present compositions may be used. The paramagnetic and superparamagnetic agents may also be coadministered separately, if desired.

The lipid and/or vesicle compositions of the present invention, and especially the vesicle compositions, may serve not only as effective carriers of the superparamagnetic agents described above, but also may improve the effect of the susceptibility contrast agents. Superparamagnetic contrast agents include metal oxides, particularly iron oxides but including manganese oxides, and as iron oxides, containing varying amounts of manganese, cobalt and nickel which experience a magnetic domain. These agents are nano or microparticles and have very high bulk susceptibilities and transverse relaxation rates. The larger particles, for example, particles having diameters of about 100 nm, have much higher R2 relaxivities as compared to R1 relaxivities. The smaller particles, for example, particles having diameters of about 10 to about 15 nm, have somewhat lower R2 relaxivities, but much more balanced R1 and R2 values. Much

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smaller particles, for example, monocrystalline iron oxide particles having diameters of about 3 to about 5 nm, have lower R2 relaxivities, but probably the most balanced R1 and R2 relaxation rates. Ferritin can also be formulated to encapsulate a core of very high relaxation rate superparamagnetic iron. It has been discovered that the lipid and/or vesicle
5 compositions, especially vesicle compositions, including gas filled vesicles, can increase the efficacy and safety of these conventional iron oxide based MRI contrast agents.

The iron oxides may simply be incorporated into the lipid and/or vesicle compositions. Preferably, in the case of vesicles formulated from lipids, the iron oxides may be incorporated into the walls of the vesicles, for example, by being adsorbed onto the
10 surfaces of the vesicles, or entrapped within the interior of the vesicles as described in U.S. Patent 5,088,499, the disclosures of which are hereby incorporated herein by reference in their entirety.

Without being bound to any particular theory or theories of operation, it is believed that the vesicles of the present invention increase the efficacy of the
15 superparamagnetic contrast agents by several mechanisms. First, it is believed that the vesicles function to increase the apparent magnetic concentration of the iron oxide particles. Also, it is believed that the vesicles increase the apparent rotational correlation time of the MRI contrast agents, including paramagnetic and superparamagnetic agents, so that relaxation rates are increased. In addition, the vesicles appear to increase the apparent
20 magnetic domain of the contrast medium according to the manner described hereinafter.

Certain of the vesicles of the present invention, and especially vesicles formulated from lipids, may be visualized as flexible spherical domains of differing susceptibility from the suspending medium, including, for example, the aqueous suspension of the contrast medium or blood or other body fluids, for example, in the case
25 of intravascular injection or injection into other body locations. In the case of ferrites or iron oxide particles, it should be noted that the contrast provided by these agents is dependent on particle size. This phenomenon is very common and is often referred to as the "secular" relaxation of the water molecules. Described in more physical terms, this relaxation mechanism is dependent upon the effective size of the molecular complex in
30 which a paramagnetic atom, or paramagnetic molecule, or molecules, may reside. One physical explanation may be described in the following Solomon-Bloembergen equations

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which define the paramagnetic contributions as a function of the T_1 and T_2 relaxation times of a spin 1/2 nucleus with gyromagnetic ratio g perturbed by a paramagnetic ion:

$$\frac{1}{T_1}M = \left(\frac{2}{15}\right) S(S+1) \gamma^2 g^2 \beta^2 / r^6 [3\tau_c/(1+\omega_l^2\tau_c^2) + \\ 7\tau_c/(1+\omega_s^2\tau_c^2)] + \left(\frac{2}{3}\right) S(S+1) A^2/h^2 [\tau_e/(1+\omega_s^2\tau_e^2)]$$

5 and

$$\frac{1}{T_2}M = \left(\frac{1}{15}\right) S(S+1) \gamma^2 g^2 \beta^2 / r^6 [4\tau_c + 3\tau_c/(1+\omega_l^2\tau_c^2) + \\ 13\tau_c/(1+\omega_s^2\tau_c^2)] + \left(\frac{1}{3}\right) S(S+1) A^2/h^2 [\tau_e/(1+\omega_s^2\tau_e^2)]$$

where:

- S is the electron spin quantum number;
- 10 g is the electronic g factor;
- β is the Bohr magneton;
- ω_l and ω_s (657 w₁) is the Larmor angular precession frequencies for the nuclear spins and electron spins;
- r is the ion-nucleus distance;
- 15 A is the hyperfine coupling constant;
- τ_c and τ_e are the correlation times for the dipolar and scalar interactions, respectively; and
- h is Planck's constant.

See, e.g., Solomon, I. *Phys. Rev.* Vol. 99, p. 559 (1955) and Bloembergen, N. *J. Chem. Phys.* Vol. 27, pp. 572, 595 (1957).

A few large particles may have a much greater effect than a larger number of much smaller particles, primarily due to a larger correlation time. If one were to make the iron oxide particles very large however, increased toxicity may result, and the lungs may be embolized or the complement cascade system may be activated. Furthermore, it is believed that the total size of the particle is not as important as the diameter of the particle at its edge or outer surface. The domain of magnetization or susceptibility effect falls off exponentially from the surface of the particle. Generally speaking, in the case of dipolar

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(through space) relaxation mechanisms, this exponential fall off exhibits an r^6 dependence for a paramagnetic dipole-dipole interaction. Interpreted literally, a water molecule that is 4 angstroms away from a paramagnetic surface will be influenced 64 times less than a water molecule that is 2 angstroms away from the same paramagnetic surface. The ideal 5 situation in terms of maximizing the contrast effect would be to make the iron oxide particles hollow, flexible and as large as possible. It has not been possible to achieve this heretofore and it is believed that the benefits have been unrecognized heretofore also. By coating the inner or outer surfaces of the vesicles with the contrast agents, even though the individual contrast agents, for example, iron oxide nanoparticles or paramagnetic ions, are 10 relatively small structures, the effectiveness of the contrast agents may be greatly enhanced. In so doing, the contrast agents may function as an effectively much larger sphere wherein the effective domain of magnetization is determined by the diameter of the vesicle and is maximal at the surface of the vesicle. These agents afford the advantage of flexibility, namely, compliance. While rigid vesicles might lodge in the lungs or other 15 organs and cause toxic reactions, these flexible vesicles slide through the capillaries much more easily.

In contrast to the flexible vesicles described above, it may be desirable, in certain circumstances, to formulate vesicles from substantially impermeable polymeric materials including, for example, polymethyl methacrylate. This would generally result in 20 the formation of vesicles which may be substantially impermeable and relatively inelastic and brittle. In embodiments involving diagnostic imaging, for example, ultrasound, contrast media which comprise such brittle vesicles would generally not provide the desirable reflectivity that the flexible vesicles may provide. However, by increasing the power output on ultrasound, the brittle microspheres can be made to rupture, thereby 25 causing acoustic emissions which can be detected by an ultrasound transducer.

Nuclear Medicine Imaging (NMI) may also be used in connection with the diagnostic and therapeutic method aspects of the present invention. For example, NMI may be used to detect radioactive gases, such as Xe^{133} , which may be incorporated in the present compositions in addition to, or instead of, the gases discussed above. Such 30 radioactive gases may be entrapped within vesicles for use in detecting, for example, thrombosis. Preferably, bifunctional chelate derivatives are incorporated in the walls of

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vesicles, and the resulting vesicles may be employed in both NMI and ultrasound. In this case, high energy, high quality nuclear medicine imaging isotopes, such as technetium^{99m} or indium¹¹¹ can be incorporated in the walls of vesicles. Whole body gamma scanning cameras can then be employed to rapidly localize regions of vesicle uptake *in vivo*. If 5 desired, ultrasound may also be used to confirm the presence, for example, of a clot within the blood vessels, since ultrasound generally provides improved resolution as compared to nuclear medicine techniques. NMI may also be used to screen the entire body of the patient to detect areas of vascular thrombosis, and ultrasound can be applied to these areas locally to promote rupture of the vesicles and treat the clot.

10 For optical imaging, optically active gases, such as argon or neon, may be incorporated in the present compositions. In addition, optically active materials, for example, fluorescent materials, including porphyrin derivatives, may also be used. Elastography is an imaging technique which generally employs much lower frequency sound, for example, about 60 KHz, as compared to ultrasound which can involve over 15 frequencies of over 1 MHz. In elastography, the sound energy is generally applied to the tissue and the elasticity of the tissue may then be determined. In connection with preferred embodiments of the invention, which involve highly elastic vesicles, the deposition of such vesicles onto, for example, a clot, increases the local elasticity of the tissue and/or the space surrounding the clot. This increased elasticity may then be detected with 20 elastography. If desired, elastography can be used in conjunction with other imaging techniques, such as MRI and ultrasound.

In addition to the aforementioned diagnostic imaging techniques and thrombolysis of clots, the present compositions can be used in a variety of therapeutic treatment modalities. For example, bioactive agents, for example, drugs, may be 25 incorporated in the present compositions. Useful drugs include, for example, heparin sulfate, tissue plasminogen activator, streptokinase, urokinase and hirudin. In connection with therapeutic applications, complementary agents can also be used including, for example, dihydroergotamine, which can be used with heparin sulfate to decrease venostasis. In addition, warfarin may be used as an adjunct to anticoagulation therapy. 30 Genetic materials may also be incorporated with the present compositions. In the case of vesicle compositions, the genetic material can be incorporated into the walls of the vesicles

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or within the central gas-filled void of the vesicles. This may be conveniently accomplished when cationic lipids are employed in the vesicle walls. Genes, such as vascular endothelial growth factor, or antisense gene fragments directed to basic fibroblast growth factor, may be used. This invention can be useful for treating underlying 5 atherosclerosis or to decrease the tendency toward fibrointimal hyperplasia.

The lipid and/or vesicle compositions of the present invention may be prepared using any of a variety of suitable methods. These are described below separately for the embodiments involving lipid compositions and a gas, including gas filled vesicles, and embodiments involving lipid compositions and a gaseous precursor, including gaseous 10 precursor filled vesicles, although compositions comprising both a gas and gaseous precursor form a part of the present invention.

A targeting ligand may be attached to the gas or gaseous precursor filled vesicle by bonding to one or more of the materials employed in the compositions from which they are made, including the lipids, proteins, polymers, and/or auxiliary stabilizing 15 materials, as described above.

A wide variety of methods are available for the preparation of the compositions, including vesicle compositions, such as micelles and/or liposomes. Included among these methods are, for example, shaking, drying, gas-installation, spray drying, and the like. Suitable methods for preparing vesicle compositions are described, for example, 20 in U.S. Patent No. 5,469,854, the disclosures of which are incorporated herein by reference. As noted above, the vesicles are preferably prepared from lipids which remain in the gel state.

With particular reference to the preparation of micelle compositions, the following discussion is provided. Micelles may be prepared using any one of a variety of 25 conventional micellar preparatory methods which will be apparent to those skilled in the art. These methods typically involve suspension of the lipid compound in an organic solvent, evaporation of the solvent, resuspension in an aqueous medium, sonication and centrifugation. The foregoing methods, as well as others, are discussed, for example, in Canfield et al., *Methods in Enzymology*, Vol. 189, pp. 418-422 (1990); El-Gorab et al, 30 *Biochem. Biophys. Acta*, Vol. 306, pp. 58-66 (1973); *Colloidal Surfactant*, Shinoda, K., Nakagana, Tamamushi and Isejura, Academic Press, NY (1963) (especially "The

Formation of Micelles", Shinoda, Chapter 1, pp. 1-88); *Catalysis in Micellar and Macromolecular Systems*, Fendler and Fendler, Academic Press, NY (1975). The disclosures of each of the foregoing publications are incorporated by reference herein, in their entirety.

5 As noted above, the vesicle composition may comprise liposomes. In any given liposome, the lipid compound(s) may be in the form of a monolayer or bilayer, and the mono- or bilayer lipids may be used to form one or more mono- or bilayers. In the case of more than one mono- or bilayer, the mono- or bilayers are generally concentric. Thus, the lipids may be used to form unilamellar liposomes (comprised of one monolayer
10 or bilayer), oligolamellar liposomes (comprised of two or three monolayers or bilayers) or multilamellar liposomes (comprised of more than three monolayers or bilayers).

A wide variety of methods are available in connection with the preparation of liposome compositions. Accordingly, the liposomes may be prepared using any one of a variety of conventional liposomal preparatory techniques which will be apparent to those skilled in the art. These techniques include, for example, solvent dialysis, French press, extrusion (with or without freeze-thaw), reverse phase evaporation, simple freeze-thaw, sonication, chelate dialysis, homogenization, solvent infusion, microemulsification, spontaneous formation, solvent vaporization, solvent dialysis, French pressure cell technique, controlled detergent dialysis, and others, each involving the preparation of the
20 vesicles in various fashions. See, e.g., Madden et al., *Chemistry and Physics of Lipids*, 1990 53, 37-46, the disclosures of which are hereby incorporated herein by reference in their entirety. Suitable freeze-thaw techniques are described, for example, in International Application Serial No. PCT/US89/05040, filed November 8, 1989, the disclosures of which are incorporated herein by reference in their entirety. Methods which involve
25 freeze-thaw techniques are preferred in connection with the preparation of liposomes. Preparation of the liposomes may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water. The liposomes may also be prepared by various processes which involve shaking or vortexing. This may be achieved, for example, by the use of a mechanical shaking device, such as a Wig-L-BugTM (Crescent
30 Dental, Lyons, IL), a Mixomat, sold by Degussa AG, Frankfurt, Germany, a Capmix, sold by Espe Fabrik Pharmazeutischer Praeparate GMBH & Co., Seefeld, Oberay Germany, a

Silamat Plus, sold by Vivadent, Lechtenstein, or a Vibros, sold by Quayle Dental, Sussex, England. Conventional microemulsification equipment, such as a Microfluidizer™ (Microfluidics, Woburn, MA) may also be used.

Spray drying may be also employed to prepare the gas-filled vesicles.

- 5 Utilizing this procedure, the lipids may be pre-mixed in an aqueous environment and then spray dried to produce gas-filled vesicles. The vesicles may be stored under a headspace of a desired gas.

Many liposomal preparatory techniques which may be adapted for use in the preparation of vesicle compositions are discussed, for example, in U.S. Patent No.

- 10 4,728,578; U.K. Patent Application GB 2193095 A; U.S. Patent No. 4,728,575; U.S. Patent No. 4,737,323; International Application Serial No. PCT/US85/01161; Mayer et al., *Biochimica et Biophysica Acta*, Vol. 858, pp. 161-168 (1986); Hope et al., *Biochimica et Biophysica Acta*, Vol. 812, pp. 55-65 (1985); U.S. Patent No. 4,533,254; Mayhew et al., *Methods in Enzymology*, Vol. 149, pp. 64-77 (1987); Mayhew et al., *Biochimica et Biophysica Acta*, Vol. 755, pp. 169-74 (1984); Cheng et al., *Investigative Radiology*, Vol. 22, pp. 47-55 (1987); International Application Serial No. PCT/US89/05040; U.S. Patent No. 4,162,282; U.S. Patent No. 4,310,505; U.S. Patent No. 4,921,706; and *Liposome Technology*, Gregoriadis, G., ed., Vol. I, pp. 29-31, 51-67 and 79-108 (CRC Press Inc., Boca Raton, FL 1984), the disclosures of each of which are hereby incorporated by reference herein, in their entirety.

Lipid compositions comprising a gas can be prepared by agitating an aqueous solution containing, if desired, a stabilizing material, in the presence of a gas. The term "agitating," as used herein, means any shaking motion of an aqueous solution such that gas is introduced from the local ambient environment into the aqueous solution.

- 25 This agitation is preferably conducted at a temperature below the gel to liquid crystalline phase transition temperature of the lipid. The shaking involved in the agitation of the solutions is preferably of sufficient force to result in the formation of a lipid composition, including vesicle compositions, and particularly vesicle compositions comprising gas filled vesicles. The shaking may be by swirling, such as by vortexing, side-to-side, or up and down motion. Different types of motion may be combined. Also, the shaking may

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occur by shaking the container holding the aqueous lipid solution, or by shaking the aqueous solution within the container without shaking the container itself.

The shaking may occur manually or by machine. Mechanical shakers that may be used include, for example, a shaker table such as a VWR Scientific (Cerritos, CA) shaker table, as well as any of the shaking devices described hereinbefore, with the Capmix (Espe Fabrik Pharmazeutischer Praeparate GMBH & Co., Seefeld, Oberbayern Germany) being preferred. It has been found that certain modes of shaking or vortexing can be used to make vesicles within a preferred size range. Shaking is preferred, and it is preferred that the shaking be carried out using the Espe Capmix mechanical shaker. In accordance with this preferred method, it is preferred that a reciprocating motion be utilized to generate the lipid compositions, and particularly vesicle compositions. It is even more preferred that the motion be reciprocating in the form of an arc. It is contemplated that the rate of reciprocation, as well as the arc thereof, is particularly important in connection with the formation of vesicles. Preferably, the number of reciprocations or full cycle oscillations is from about 1000 to about 20,000 per minute. More preferably, the number of reciprocations or oscillations is from about 2500 to about 8000, with reciprocations or oscillations of from about 3300 to about 5000 being even more preferred. Of course, the number of oscillations can be dependent upon the mass of the contents being agitated. Generally speaking, a larger mass requires fewer oscillations.

Another means for producing shaking includes the action of gas emitted under high velocity or pressure.

It will also be understood that preferably, with a larger volume of aqueous solution, the total amount of force will be correspondingly increased. Vigorous shaking is defined as at least about 60 shaking motions per minute, and is preferred. Vortexing at about 60 to about 300 revolutions per minute is more preferred. Vortexing at about 300 to about 1800 revolutions per minute is even more preferred.

In addition to the simple shaking methods described above, more elaborate methods can also be employed. Such elaborate methods include, for example, liquid crystalline shaking gas instillation processes and vacuum drying gas instillation processes, such as those described in copending U.S. application Serial No. 08/076,250, filed June 11, 1993, the disclosures of which are incorporated herein by reference, in their entirety.

Although any of a number of varying techniques can be used, the vesicle compositions employed in the present invention are preferably prepared using a shaking technique. Preferably, the shaking technique involves agitation with a mechanical shaking apparatus, such as an Espe Capmix (Seefeld, Oberay Germany), using, for example, the techniques 5 disclosed in copending U.S. application Serial No. 160,232, filed November 30, 1993, the disclosures of which are hereby incorporated herein by reference in their entirety.

The size of gas filled vesicles can be adjusted, if desired, by a variety of procedures, including, for example, microemulsification, vortexing, extrusion, filtration, sonication, homogenization, repeated freezing and thawing cycles, extrusion under 10 pressure through pores of defined size, and similar methods. Gas filled vesicles prepared in accordance with the methods described herein can range in size from less than about 1 μm to greater than about 100 μm . In addition, after extrusion and sterilization procedures, which are discussed in detail below, agitation or shaking provides vesicle compositions which provide substantially no or minimal residual anhydrous lipid phase in the remainder 15 of the solution. (Bangham, A.D., Standish, M.M., & Watkins, J.C., *J. Mol. Biol.* Vol. 13, pp. 238-252 (1965). If desired, the vesicles of the present invention may be used as they are formed, without any attempt at further modification of the size thereof. For intravascular use, the vesicles preferably have diameters of less than about 30 μm , and more preferably, less than about 12 μm . For targeted intravascular use including, for 20 example, binding to certain tissue, such as cancerous tissue, the vesicles can be significantly smaller, for example, less than about 100 nm in diameter. For enteric or gastrointestinal use, the vesicles can be significantly larger, for example, up to a millimeter in size. Preferably, the vesicles are sized to have diameters of from about 2 μm to about 100 μm .

25 The gas filled vesicles may be sized by a simple process of extrusion through filters wherein the filter pore sizes control the size distribution of the resulting gas filled vesicles. By using two or more cascaded or stacked set of filters, for example, a 10 μm filter followed by an 8 μm filter, the gas filled vesicles can be selected to have a very narrow size distribution around 7 to 9 μm . After filtration, these gas filled vesicles can 30 remain stable for over 24 hours.

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The sizing or filtration step may be accomplished by the use, for example, of a filter assembly when the composition is removed from a sterile vial prior to use, or more preferably, the filter assembly may be incorporated into a syringe during use. The method of sizing the vesicles will then comprise using a syringe comprising a barrel, at 5 least one filter, and a needle; and will be carried out by a step of extracting which comprises extruding the vesicles from the barrel through the filter fitted to the syringe between the barrel and the needle, thereby sizing the vesicles before they are administered to a patient. The step of extracting may also comprise drawing the vesicles into the 10 syringe, where the filter will function in the same way to size the vesicles upon entrance into the syringe. Another alternative is to fill such a syringe with vesicles which have already been sized by some other means, in which case the filter now functions to ensure that only vesicles within the desired size range, or of the desired maximum size, are subsequently administered by extrusion from the syringe.

In certain preferred embodiments, the vesicle compositions may be heat 15 sterilized or filter sterilized and extruded through a filter prior to shaking. Generally speaking, vesicle compositions comprising a gas may be heat sterilized, and vesicle compositions comprising gaseous precursors may be filter sterilized. Once gas filled vesicles are formed, they may be filtered for sizing as described above. Performing these 20 steps prior to the formation of gas and gaseous precursor filled vesicles provide sterile gas filled vesicles ready for administration to a patient. For example, a mixing vessel such as a vial or syringe may be filled with a filtered lipid composition, and the composition may be sterilized within the mixing vessel, for example, by autoclaving. Gas may be instilled into the composition to form gas filled vesicles by shaking the sterile vessel. Preferably, the 25 sterile vessel is equipped with a filter positioned such that the gas filled vesicles pass through the filter before contacting a patient.

The step of extruding the solution of lipid compound through a filter decreases the amount of unhydrated material by breaking up any dried materials and exposing a greater surface area for hydration. Preferably, the filter has a pore size of about 0.1 to about 5 μm , more preferably, about 0.1 to about 4 μm , even more preferably, about 30 0.1 to about 2 μm , and still more preferably, about 1 μm . Unhydrated compound, which is generally undesirable, appears as amorphous clumps of non-uniform size.

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The sterilization step provides a composition that may be readily administered to a patient for diagnostic imaging including, for example, ultrasound or CT. In certain preferred embodiments, sterilization may be accomplished by heat sterilization, preferably, by autoclaving the solution at a temperature of at least about 100°C, and more 5 preferably, by autoclaving at about 100°C to about 130°C, even more preferably, about 110°C to about 130°C, still more preferably, about 120°C to about 130°C, and even more preferably, about 130°C. Preferably, heating occurs for at least about 1 minute, more preferably, about 1 to about 30 minutes, even more preferably, about 10 to about 20 minutes, and still more preferably, about 15 minutes.

10 If desired, the extrusion and heating steps, as outlined above, may be reversed, or only one of the two steps can be used. Other modes of sterilization may be used, including, for example, exposure to gamma radiation.

In addition to the aforementioned embodiments, gaseous precursors contained in vesicles can be formulated which, upon activation, for example, by exposure 15 to elevated temperature, varying pH, or light, undergo a phase transition from, for example, a liquid, including a liquid entrapped in a vesicle, to a gas, expanding to create the gas filled vesicles described herein. This technique is described in detail in copending patent applications Serial Nos. 08/160,232, filed November 30, 1993 and 08/159,687, filed November 30, 1993, the disclosures of which are incorporated herein by reference, in 20 their entirety.

The preferred method of activating the gaseous precursor is by exposure to elevated temperature. Activation or transition temperature, and like terms, refer to the boiling point of the gaseous precursor and is the temperature at which the liquid to gaseous phase transition of the gaseous precursor takes place. Useful gaseous precursors are those 25 materials which have boiling points in the range of about -100°C to about 70°C. The activation temperature is particular to each gaseous precursor. An activation temperature of about 37°C, or about human body temperature, is preferred for gaseous precursors in the context of the present invention. Thus, in preferred form, a liquid gaseous precursor is activated to become a gas at about 37°C or below. The gaseous precursor may be in liquid 30 or gaseous phase for use in the methods of the present invention.

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The methods of preparing the gaseous precursor filled vesicles may be carried out below the boiling point of the gaseous precursor such that a liquid is incorporated, for example, into a vesicle. In addition, the methods may be conducted at the boiling point of the gaseous precursor, such that a gas is incorporated, for example,

5 into a vesicle. For gaseous precursors having low temperature boiling points, liquid precursors may be emulsified using a microfluidizer device chilled to a low temperature. The boiling points may also be depressed using solvents in liquid media to utilize a precursor in liquid form. Further, the methods may be performed where the temperature is increased throughout the process, whereby the process starts with a gaseous precursor as a

10 liquid and ends with a gas.

The gaseous precursor may be selected so as to form the gas *in situ* in the targeted tissue or fluid, *in vivo* upon entering the patient or animal, prior to use, during storage, or during manufacture. The methods of producing the temperature-activated gaseous precursor filled vesicles may be carried out at a temperature below the boiling

15 point of the gaseous precursor. In this embodiment, the gaseous precursor is entrapped within a vesicle such that the phase transition does not occur during manufacture. Instead, the gaseous precursor filled vesicles are manufactured in the liquid phase of the gaseous precursor. Activation of the phase transition may take place at any time as the temperature is allowed to exceed the boiling point of the precursor. Also, knowing the amount of

20 liquid in a droplet of liquid gaseous precursor, the size of the vesicles upon attaining the gaseous state may be determined.

Alternatively, the gaseous precursors may be utilized to create stable gas filled vesicles which are pre-formed prior to use. In this embodiment, the gaseous precursor is added to a container housing a lipid composition at a temperature below the

25 liquid-gaseous phase transition temperature of the respective gaseous precursor. As the temperature is increased, and an emulsion is formed between the gaseous precursor and liquid solution, the gaseous precursor undergoes transition from the liquid to the gaseous state. As a result of this heating and gas formation, the gas displaces the air in the head space above the liquid mixture so as to form gas filled vesicles which entrap the gas of the

30 gaseous precursor, ambient gas (e.g. air), or coentrain gas state gaseous precursor and ambient air. This phase transition can be used for optimal mixing and formation of the

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contrast agent. For example, the gaseous precursor, perfluorobutane, can be entrapped in the lipid vesicles and as the temperature is raised beyond the boiling point of perfluorobutane (4°C), perfluorobutane gas is entrapped in the vesicles.

Accordingly, the gaseous precursors may be selected to form gas filled vesicles *in vivo* or may be designed to produce the gas filled vesicles *in situ*, during the manufacturing process, on storage, or at some time prior to use. A water bath, sonicator or hydrodynamic activation by pulling back the plunger of a syringe against a closed stopcock may be used to activate targeted gas-filled vesicles from temperative-sensitive gaseous precursors prior to I.V. injection.

As a further embodiment of this invention, by pre-forming the gaseous precursor in the liquid state into an aqueous emulsion, the maximum size of the vesicle may be estimated by using the ideal gas law, once the transition to the gaseous state is effectuated. For the purpose of making gas filled vesicles from gaseous precursors, the gas phase is assumed to form instantaneously and substantially no gas in the newly formed vesicle has been depleted due to diffusion into the liquid, which is generally aqueous in nature. Hence, from a known liquid volume in the emulsion, one would be able to predict an upper limit to the size of the gas filled vesicle.

In embodiments of the present invention, a mixture of a lipid compound and a gaseous precursor, containing liquid droplets of defined size, may be formulated such that upon reaching a specific temperature, for example, the boiling point of the gaseous precursor, the droplets will expand into gas filled vesicles of defined size. The defined size represents an upper limit to the actual size because the ideal gas law cannot account for such factors as gas diffusion into solution, loss of gas to the atmosphere, and the effects of increased pressure.

The ideal gas law, which can be used for calculating the increase in the volume of the gas bubbles upon transitioning from liquid to gaseous states, is as follows:

$$PV = nRT$$

where

P is pressure in atmospheres (atm);

V is volume in liters (L);

n is moles of gas;

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T is temperature in degrees Kelvin (K); and

R is the ideal gas constant (22.4 L-atm/K-mole).

With knowledge of volume, density, and temperature of the liquid in the mixture of liquids, the amount, for example, in moles, and volume of liquid precursor may be
 5 calculated which, when converted to a gas, will expand into a vesicle of known volume. The calculated volume will reflect an upper limit to the size of the gas filled vesicle, assuming instantaneous expansion into a gas filled vesicle and negligible diffusion of the gas over the time of the expansion.

Thus, for stabilization of the precursor in the liquid state in a mixture
 10 wherein the precursor droplet is spherical, the volume of the precursor droplet may be determined by the equation:

$$\text{Volume (spherical vesicle)} = \frac{4}{3} \pi r^3$$

where

r is the radius of the sphere.

15 Thus, once the volume is predicted, and knowing the density of the liquid at the desired temperature, the amount of liquid gaseous precursor in the droplet may be determined. In more descriptive terms, the following can be applied:

$$V_{\text{gas}} = \frac{4}{3} \pi (r_{\text{gas}})^3$$

by the ideal gas law,

$$PV=nRT$$

substituting reveals,

$$V_{\text{gas}} = nRT/P_{\text{gas}}$$

or,

$$(A) \quad n = \frac{4}{3} [\pi r_{\text{gas}}^3] P/RT$$

$$25 \quad \text{amount } n = \frac{4}{3} [\pi r_{\text{gas}}^3 P/RT] \cdot MW_n$$

Converting back to a liquid volume

$$(B) \quad V_{\text{liq}} = [4/3 [\pi r_{\text{gas}}^3] P/RT] \cdot MW_n/D$$

where D is the density of the precursor.

Solving for the diameter of the liquid droplet,

$$30 \quad (C) \quad \text{diameter}/2 = [3/4\pi [4/3 \cdot [\pi r_{\text{gas}}^3] P/RT] MW_n/D]^{1/3}$$

which reduces to

$$\text{Diameter} = 2[[r_{\text{gas}}^3] P/RT [MW_r/D]]^{1/3}.$$

As a further means of preparing vesicles of the desired size for use in the methods of the present invention, and with a knowledge of the volume and especially the 5 radius of the liquid droplets, one can use appropriately sized filters to size the gaseous precursor droplets to the appropriate diameter sphere.

A representative gaseous precursor may be used to form a vesicle of defined size, for example, 10 μm diameter. In this example, the vesicle is formed in the bloodstream of a human being, thus the typical temperature would be 37°C or 10 310 K. At a pressure of 1 atmosphere and using the equation in (A), 7.54×10^{-17} moles of gaseous precursor would be required to fill the volume of a 10 μm diameter vesicle.

Using the above calculated amount of gaseous precursor and 1-fluorobutane, which possesses a molecular weight of 76.11, a boiling point of 32.5°C and a density of 0.7789 g/mL at 20°C, further calculations predict that 15 5.74×10^{-15} grams of this precursor would be required for a 10 μm vesicle. Extrapolating further, and with the knowledge of the density, equation (B) further predicts that 8.47×10^{-16} mL of liquid precursor is necessary to form a vesicle with an upper limit of 10 μm .

Finally, using equation (C), a mixture, for example, an emulsion containing droplets with a radius of 0.0272 μm or a corresponding diameter of 20 0.0544 μm , is formed to make a gaseous precursor filled vesicle with an upper limit of a 10 μm vesicle.

An emulsion of this particular size could be easily achieved by the use of an appropriately sized filter. In addition, as seen by the size of the filter necessary to form 25 gaseous precursor droplets of defined size, the size of the filter would also suffice to remove any possible bacterial contaminants and, hence, can be used as a sterile filtration as well.

This embodiment for preparing gas filled vesicles may be applied to all gaseous precursors activated by temperature. In fact, depression of the freezing point of the solvent system allows the use of gaseous precursors which would undergo liquid-to- 30 gas phase transitions at temperatures below 0°C. The solvent system can be selected to provide a medium for suspension of the gaseous precursor. For example, 20% propylene

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glycol miscible in buffered saline exhibits a freezing point depression well below the freezing point of water alone. By increasing the amount of propylene glycol or adding materials such as sodium chloride, the freezing point can be depressed even further.

The selection of appropriate solvent systems may be determined by

- 5 physical methods as well. When substances, solid or liquid, herein referred to as solutes, are dissolved in a solvent, such as water based buffers, the freezing point is lowered by an amount that is dependent upon the composition of the solution. Thus, as defined by Wall, one can express the freezing point depression of the solvent by the following equation:

$$\ln x_a = \ln (1 - x_b) = \Delta H_{fus}/R(1/T_o - 1/T)$$

10 where

x_a is the mole fraction of the solvent;

x_b is the mole fraction of the solute;

ΔH_{fus} is the heat of fusion of the solvent; and

T_o is the normal freezing point of the solvent.

- 15 The normal freezing point of the solvent can be obtained by solving the equation. If x_b is small relative to x_a , then the above equation may be rewritten as follows.

$$x^b = \Delta H_{fus}/R[T - T_o/T_o] \approx \Delta H_{fus}\Delta T/RT_o^2$$

The above equation assumes the change in temperature ΔT is small compared to T_o . This equation can be simplified further by expressing the concentration of the solute in terms of

- 20 molality, m (moles of solute per thousand grams of solvent). Thus, the equation can be rewritten as follows.

$$X_b = m/[m + 1000/m_a] \approx mM_a/1000$$

where M_a is the molecular weight of the solvent.

Thus, substituting for the fraction x_b :

25 $\Delta T = [M_a RT_o^2/1000\Delta H_{fus}]m$

or

$$\Delta T = K_f m, \text{ where}$$

$$K_f = M_a RT_o^2/1000\Delta H_{fus}$$

K_f is the molal freezing point and is equal to 1.86 degrees per unit of molal concentration

- 30 for water at one atmosphere pressure. The above equation may be used to accurately determine the molal freezing point of solutions of gaseous-precursor filled vesicles.

Accordingly, the above equation can be applied to estimate freezing point depressions and to determine the appropriate concentrations of liquid or solid solute necessary to depress the solvent freezing temperature to an appropriate value.

Methods of preparing the temperature activated gaseous precursor filled
5 vesicles include:

- (a) vortexing and/or shaking an aqueous mixture of gaseous precursor and additional materials as desired, including, for example, stabilizing materials, thickening agents and/or dispersing agents. Optional variations of this method include autoclaving before vortexing or shaking; heating an aqueous mixture of gaseous precursor; venting the
10 vessel containing the mixture/suspension; shaking or permitting the gaseous precursor filled vesicle to form spontaneously and cooling down the suspension of gaseous precursor filled vesicles; and extruding an aqueous suspension of gaseous precursor through a filter of about 0.22 µm. Alternatively, filtering may be performed during *in vivo* administration of the vesicles such that a filter of about 0.22 µm is employed;
- 15 (b) microemulsification whereby an aqueous mixture of gaseous precursor is emulsified by agitation and heated to form, for example, vesicles prior to administration to a patient;
- (c) heating a gaseous precursor in a mixture, with or without agitation, whereby the less dense gaseous precursor filled vesicles float to the top of the solution by
20 expanding and displacing other vesicles in the vessel and venting the vessel to release air; and
- (d) utilizing in any of the above methods a sealed vessel to hold the aqueous suspension of gaseous precursor and maintaining the suspension at a temperature below the phase transition temperature of the gaseous precursor, followed by autoclaving to raise
25 the temperature above the phase transition temperature, optionally with shaking, or permitting the gaseous precursor vesicle to form spontaneously, whereby the expanded gaseous precursor in the sealed vessel increases the pressure in the vessel, and cooling down the gas filled vesicle suspension, after which shaking may also take place.

Freeze drying is useful to remove water and organic materials prior to the
30 shaking installation method. Drying installation methods may be used to remove water from vesicles. By pre-entrapping the gaseous precursor in the dried vesicles (i.e. prior to

drying) after warming, the gaseous precursor may expand to fill the vesicle. Gaseous precursors can also be used to fill dried vesicles after they have been subjected to vacuum. As the dried vesicles are kept at a temperature below their gel state to liquid crystalline temperature, the drying chamber can be slowly filled with the gaseous precursor in its 5 gaseous state. For example, perfluorobutane can be used to fill dried vesicles at temperatures above 4 °C (the boiling point of perfluorobutane).

Preferred methods for preparing the temperature activated gaseous precursor filled vesicles comprise shaking an aqueous solution having a lipid compound in the presence of a gaseous precursor at a temperature below the liquid state to gas state 10 phase transition temperature of the gaseous precursor. This is preferably conducted at a temperature below the gel state to liquid crystalline state phase transition temperature of the lipid. The mixture is then heated to a temperature above the liquid state to gas state phase transition temperature of the gaseous precursor which causes the precursor to volatilize and expand. Heating is then discontinued, and the temperature of the mixture is 15 then allowed to drop below the liquid state to gas state phase transition temperature of the gaseous precursor. Shaking of the mixture may take place during the heating step, or subsequently after the mixture is allowed to cool.

Other methods for preparing gaseous precursor filled vesicles can involve shaking an aqueous solution of, for example, a lipid and a gaseous precursor, and 20 separating the resulting gaseous precursor filled vesicles.

Conventional, aqueous-filled liposomes of the prior art are routinely formed at a temperature above the phase transition temperature of the lipids used to make them, since they are more flexible and thus useful in biological systems in the liquid crystalline state. See, for example, Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci.* 1978, 75, 25 4194-4198. In contrast, the vesicles made according to certain preferred embodiments described herein are gaseous precursor filled, which imparts greater flexibility, since gaseous precursors after gas formation are more compressible and compliant than an aqueous solution.

The methods contemplated by the present invention provide for shaking an 30 aqueous solution comprising a lipid, in the presence of a temperature activatable gaseous precursor. Preferably, the shaking is of sufficient force such that a foam is formed within a

short period of time, such as about 30 minutes, and preferably within about 20 minutes, and more preferably, within about 10 minutes. The shaking may involve microemulsifying, microfluidizing, swirling (such as by vortexing), side-to-side, or up and down motion. In the case of the addition of gaseous precursor in the liquid state,

5 sonication may be used in addition to the shaking methods set forth above. Further, different types of motion may be combined. Also, the shaking may occur by shaking the container holding the aqueous lipid solution, or by shaking the aqueous solution within the container without shaking the container itself. Further, the shaking may occur manually or by machine. Mechanical shakers that may be used include, for example, the mechanical

10 shakers described hereinbefore, with an Espe Capmix (Seefeld, Oberay Germany) being preferred. Another means for producing shaking includes the action of gaseous precursor emitted under high velocity or pressure.

According to the methods described herein, a gas, such as air, may also be provided by the local ambient atmosphere. The local ambient atmosphere can include the

15 atmosphere within a sealed container, as well as the external environment. Alternatively, for example, a gas may be injected into or otherwise added to the container having the aqueous lipid solution or into the aqueous lipid solution itself to provide a gas other than air. Gases that are lighter than air are generally added to a sealed container, while gases heavier than air can be added to a sealed or an unsealed container. Accordingly, the

20 present invention includes co-entrapment of air and/or other gases along with gaseous precursors.

Hence, the gaseous precursor filled vesicles can be used in substantially the same manner as the gas filled vesicles described herein, once activated by application to the tissues of a host, where such factors as temperature or pH may be used to cause

25 generation of the gas. It is preferred that the gaseous precursors undergo phase transitions from liquid to gaseous states at near the normal body temperature of the host, and are thereby activated, for example, by the *in vivo* temperature of the host so as to undergo transition to the gaseous phase therein. Alternating, activation prior to I.V. injection may be used, for example, by thermal, mechanical or optical means. This activation can occur

30 where, for example, the host tissue is human tissue having a normal temperature of about

37°C and the gaseous precursors undergo phase transitions from liquid to gaseous states near 37°C.

As noted above, the lipid and/or vesicle compositions may be sterilized by autoclave or sterile filtration if these processes are performed before the installation step or 5 prior to temperature mediated conversion of the temperature sensitive gaseous precursors within the compositions. Alternatively, one or more anti-bactericidal agents and/or preservatives may be included in the formulation of the compositions, such as sodium benzoate, quaternary ammonium salts, sodium azide, methyl paraben, propyl paraben, sorbic acid, ascorbylpalmitate, butylated hydroxyanisole, butylated hydroxytoluene, 10 chlorobutanol, dehydroacetic acid, ethylenediamine, monothioglycerol, potassium benzoate, potassium metabisulfite, potassium sorbate, sodium bisulfite, sulfur dioxide, and organic mercurial salts. Such sterilization, which may also be achieved by other conventional means, such as by irradiation, will be necessary where the stabilized vesicles are used for imaging under invasive circumstances, e.g., intravascularly or 15 intraperitoneally. The appropriate means of sterilization will be apparent to the artisan based on the present disclosure.

Vesicle compositions which comprise vesicles formulated from proteins (also referred to as protein encapsulated microbubbles), such as albumin vesicles, may be prepared by various processes, as will be readily apparent to those skilled in the art, once 20 armed with the present disclosure. Suitable methods include those described, for example, in Feinstein, U.S. Patent Nos. 4,572,203, 4,718,433 and 4,774,958, and Cerny et al., U.S. Patent No. 4,957,656, the disclosures of which are hereby incorporated herein by reference, in their entirety. Included among the methods described in the aforementioned 25 patents for the preparation of protein-based vesicles are methods which involve sonicating a solution of a protein. In preferred form, the starting material may be an aqueous solution of a heat-denaturable, water-soluble biocompatible protein. The encapsulating protein is preferably heat-sensitive so that it can be partially insolubilized by heating during sonication. Suitable heat-sensitive proteins include, for example, albumin, hemoglobin, collagen, and the like. Preferably, the protein is a human protein, with human serum 30 albumin (HSA) being more preferred. HSA is available commercially as a sterile 5% aqueous solution, which is suitable for use in the preparation of protein-based vesicles. Of

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course, as would be apparent to one of ordinary skill in the art, other concentrations of albumin, as well as other proteins which are heat-denaturable, can be used to prepare the vesicles. Generally speaking, the concentration of HSA can vary and may range from about 0.1 to about 25% by weight, and all combinations and subcombinations of ranges 5 therein. It may be preferable, in connection with certain methods for the preparation of protein-based vesicles, to utilize the protein in the form of a dilute aqueous solution. For albumin, it may be preferred to utilize an aqueous solution containing from about 0.5 to about 7.5% by weight albumin, with concentrations of less than about 5% by weight being preferred, for example, from about 0.5 to about 3% by weight.

10 The protein-based vesicles may be prepared using equipment which is commercially available. For example, in connection with a feed preparation operation as disclosed, for example, in Cerny, et al., U.S. Patent No. 4,957,656, stainless steel tanks which are commercially available from Walker Stainless Equipment Co. (New Lisbon, WI), and process filters which are commercially available from Millipore (Bedford, MA), 15 may be utilized.

 The sonication operation may utilize both a heat exchanger and a flow through sonicating vessel, in series. Heat exchanger equipment of this type may be obtained from ITT Standard (Buffalo, NY). The heat exchanger maintains operating temperature for the sonication process, with temperature controls ranging from about 65°C 20 to about 80°C, depending on the makeup of the media. The vibration frequency of the sonication equipment may vary over a wide range, for example, from about 5 to about 40 kilohertz (kHz), with a majority of the commercially available sonicators operating at about 10 or 20 kHz. Suitable sonicating equipment include, for example, a Sonics & Materials Vibra-Cell, equipped with a flat-tipped sonicator horn, commercially available 25 from Sonics & Materials, Inc. (Danbury, CT). The power applied to the sonicator horn can be varied over power settings scaled from 1 to 10 by the manufacturer, as with Sonics & Materials Vibra-Cell Model VL1500. An intermediate power setting, for example, from 5 to 9, can be used. It is preferred that the vibrational frequency and the power supplied be sufficient to produce cavitation in the liquid being sonicated. Feed flow rates may range 30 from about 50 mL/min to about 1000 mL/min, and all combinations and subcombinations of ranges therein. Residence times in the sonication vessel can range from about 1 second

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to about 4 minutes, and gaseous fluid addition rates may range from about 10 cubic centimeters (cc) per minute to about 100 cc/min, or 5% to 25% of the feed flow rate, and all combinations and subcombinations of ranges therein.

It may be preferable to carry out the sonication in such a manner to produce 5 foaming, and especially intense foaming, of the solution. Generally speaking, intense foaming and aerosolating are important for obtaining a contrast agent having enhanced concentration and stability. To promote foaming, the power input to the sonicator horn may be increased, and the process may be operated under mild pressure, for example, about 1 to about 5 psi. Foaming may be easily detected by the cloudy appearance of the 10 solution, and by the foam produced.

Suitable methods for the preparation of protein-based vesicles may also involve physically or chemically altering the protein or protein derivative in aqueous solution to denature or fix the material. For example, protein-based vesicles may be prepared from a 5% aqueous solution of HSA by heating after formation or during 15 formation of the contrast agent via sonication. Chemical alteration may involve chemically denaturing or fixing by binding the protein with a difunctional aldehyde, such as gluteraldehyde. For example, the vesicles may be reacted with 0.25 grams of 50% aqueous gluteraldehyde per gram of protein at pH 4.5 for 6 hours. The unreacted gluteraldehyde may then be washed away from the protein.

20 In any of the techniques described above for the preparation of protein-based vesicles, the targeting ligands may be incorporated with the proteins before, during or after formation of the vesicles, as would be apparent to one of ordinary skill in the art, once armed with the present disclosure.

Vesicle compositions which comprise vesicles formulated from polymers 25 may be prepared by various processes, as will be readily apparent to those skilled in the art, once armed with the present disclosure. Exemplary processes include, for example, interfacial polymerization, phase separation and coacervation, multiorifice centrifugal preparation, and solvent evaporation. Suitable procedures which may be employed or modified in accordance with the present disclosure to prepare vesicles from polymers 30 include those procedures disclosed in Garner et al., U.S. Patent No. 4,179,546, Garner, U.S. Patent No. 3,945,956, Cohrs et al., U.S. Patent No. 4,108,806, Japan Kokai Tokkyo

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- Koho 62 286534, British Patent No. 1,044,680, Kenaga et al., U.S. Patent No. 3,293,114, Morehouse et al., U.S. Patent No. 3,401,475, Walters, U.S. Patent No. 3,479,811, Walters et al., U.S. Patent No. 3,488,714, Morehouse et al., U.S. Patent No. 3,615,972, Baker et al., U.S. Patent No. 4,549,892, Sands et al., U.S. Patent No. 4,540,629, Sands et al., U.S.
- 5 Patent No. 4,421,562, Sands, U.S. Patent No. 4,420,442, Mathiowitz et al., U.S. Patent No. 4,898,734, Lencki et al., U.S. Patent No. 4,822,534, Herbig et al., U.S. Patent No. 3,732,172, Himmel et al., U.S. Patent No. 3,594,326, Sommerville et al., U.S. Patent No. 3,015,128, Deasy, *Microencapsulation and Related Drug Processes*, Vol. 20, Chs. 9 and 10, pp. 195-240 (Marcel Dekker, Inc., N.Y., 1984), Chang et al., *Canadian J. of 10 Physiology and Pharmacology*, Vol 44, pp. 115-129 (1966), and Chang, *Science*, Vol. 146, pp. 524-525 (1964), the disclosures of each of which are incorporated herein by reference in their entirety.

In accordance with a preferred synthesis protocol, the vesicles may be prepared using a heat expansion process, such as, for example, the process described in

15 Garner et al., U.S. Patent No. 4,179,546, Garner, U.S. Patent No. 3,945,956, Cohrs et al., U.S. Patent No. 4,108,806, British Patent No. 1,044,680, and Japan Kokai Tokkyo Koho 62 286534. In general terms, the heat expansion process may be carried out by preparing vesicles of an expandable polymer or copolymer which may contain in their void (cavity) a volatile liquid (gaseous precursor). The vesicle is then heated, plasticising the vesicle and

20 converting the volatile liquid into a gas, causing the vesicle to expand to up to about several times its original size. When the heat is removed, the thermoplastic polymer retains at least some of its expanded shape. Vesicles produced by this process tend to be of particularly low density, and are thus preferred. The foregoing described process is well known in the art, and may be referred to as the heat expansion process for preparing low

25 density vesicles.

Polymers useful in the heat expansion process will be readily apparent to those skilled in the art and include thermoplastic polymers or copolymers, including polymers or copolymers of many of the monomers described above. Preferable of the polymers and copolymers described above include the following copolymers:

30 polyvinylidene-polyacrylonitrile, polyvinylidene-polyacrylonitrile-polymethyl-

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methacrylate, and polystyrene-polyacrylonitrile. A most preferred copolymer is polyvinylidene-polyacrylonitrile.

Volatile liquids useful in the heat expansion process will also be well known to those skilled in the art and include: aliphatic hydrocarbons such as ethane, 5 ethylene, propane, propene, butane, isobutane, neopentane, acetylene, hexane, heptane; chlorofluorocarbons such as CCl_3F , CCl_2F_3 , CClF_3 , $\text{CClF}_2\text{-CCl}_2\text{F}_2$, chloroheptafluoro-cyclobutane, and 1,2-dichlorohexafluorocyclobutane; tetraalkyl silanes, such as tetramethyl silane, trimethylethyl silane, trimethylisopropyl silane, and trimethyl n-propyl silane; as well as perfluorocarbons, including the perfluorocarbons described above. In 10 general, it is important that the volatile liquid not be a solvent for the polymer or copolymer being utilized. It is also preferred that the volatile liquid have a boiling point that is below the softening point of the involved polymer or co-polymer. Boiling points of various volatile liquids and softening points of various polymers and copolymers will be readily ascertainable to one skilled in the art, and suitable combinations of polymers or 15 copolymers and volatile liquids will be easily apparent to the skilled artisan. By way of guidance, and as one skilled in the art would recognize, generally as the length of the carbon chain of the volatile liquid increases, the boiling point of that liquid increases also. Also, mildly preheating the vesicles in water in the presence of hydrogen peroxide prior to definitive heating and expansion may pre-soften the vesicle to allow expansion to occur 20 more readily.

For example, to produce vesicles from synthetic polymers, vinylidene and acrylonitrile may be copolymerized in a medium of isobutane liquid using one or more of the foregoing modified or unmodified literature procedures, such that isobutane becomes entrapped within the vesicles. When such vesicles are then heated to a temperature of 25 from about 80°C to about 120°C, the isobutane gas expands, which in turn expands the vesicles. After heat is removed, the expanded polyvinylidene and acrylo-nitrile copolymer vesicles remain substantially fixed in their expanded position. The resulting low density vesicles are extremely stable both dry and suspended in an aqueous media. Isobutane is utilized herein merely as an illustrative liquid, with the understanding that other liquids 30 which undergo liquid/gas transitions at temperatures useful for the synthesis of these vesicles and formation of the very low density vesicles upon heating can be substituted for

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isobutane. Similarly, monomers other than vinylidene and acrylonitrile may be employed in preparing the vesicles.

In certain preferred embodiments, the vesicles which are formulated from synthetic polymers and which may be employed in the methods of the present invention 5 are commercially available from Expance, Nobel Industries (Sundsvall, Sweden), including EXPANCEL 551 DETM microspheres. The EXPANCEL 551 DETM microspheres are composed of a copolymer of vinylidene and acrylonitrile which have encapsulated therein isobutane liquid. Such microspheres are sold as a dry composition and are approximately 50 microns in size. The EXPANCEL 551 DETM microspheres have 10 a specific gravity of only 0.02 to 0.05, which is between one-fiftieth and one-twentieth the density of water.

In any of the techniques described above for the preparation of polymer-based vesicles, the targeting ligands may be incorporated with the polymers before, during or after formation of the vesicles, as would be apparent to one of ordinary skill in the art, 15 once armed with the present disclosure.

As with the preparation of lipid and/or vesicle compositions, a wide variety of techniques are available for the preparation of lipid formulations. For example, the lipid and/or vesicle formulations may be prepared from a mixture of lipid compounds, bioactive agent and gas or gaseous precursor. In this case, lipid compositions are prepared as 20 described above in which the compositions also comprise bioactive agent. Thus, for example, micelles can be prepared in the presence of a bioactive agent. In connection with lipid compositions which comprise a gas, the preparation can involve, for example, bubbling a gas directly into a mixture of the lipid compounds and one or more additional materials. Alternatively, the lipid compositions may be preformed from lipid compounds 25 and gas or gaseous precursor. In the latter case, the bioactive agent is then added to the lipid composition prior to use. For example, an aqueous mixture of liposomes and gas may be prepared to which the bioactive agent is added and which is agitated to provide the liposome formulation. The liposome formulation can be readily isolated since the gas and/or bioactive agent filled liposome vesicles generally float to the top of the aqueous 30 solution. Excess bioactive agent can be recovered from the remaining aqueous solution.

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- As those skilled in the art will recognize, any of the lipid and/or vesicle compositions and/or lipid and/or vesicle formulations may be lyophilized for storage, and reconstituted, for example, with an aqueous medium (such as sterile water, phosphate buffered solution, or aqueous saline solution), with the aid of vigorous agitation. To prevent agglutination or fusion of the lipids and/or vesicles as a result of lyophilization, it may be useful to include additives which prevent such fusion or agglutination from occurring. Additives which may be useful include sorbitol, mannitol, sodium chloride, glucose, trehalose, polyvinylpyrrolidone and poly(ethylene glycol) (PEG), for example, PEG 400. These and other additives are described in the literature, such as in the U.S. Pharmacopeia, USP XXII, NF XVII, The United States Pharmacopeia, The National Formulary, United States Pharmacopeial Convention Inc., 12601 Twinbrook Parkway, Rockville, MD 20852, the disclosures of which are hereby incorporated herein by reference in their entirety. Lyophilized preparations generally have the advantage of greater shelf life.
- As discussed above, the compositions of the present invention, including gas and/or gaseous precursor filled vesicles, are useful as contrast agents for diagnostic imaging, including, for example, ultrasound imaging (US), computed tomography (CT) imaging, including CT angiography (CTA) imaging, magnetic resonance (MR) imaging, magnetic resonance angiography (MRA), nuclear medicine, optical imaging and elastography.
- In accordance with the present invention, there are provided methods of imaging one or more regions of a patient. The present invention also provides methods for diagnosing the presence or absence of diseased tissue in a patient. The methods of the present invention involve the administration of a contrast medium, in the form of a lipid and/or vesicle composition, to a patient. The patient is scanned using diagnostic imaging including, for example ultrasound imaging, to obtain visible images of an internal region of a patient. The methods are especially useful in providing images of the heart region, the gastrointestinal region or the lymphatic system, but can also be employed more broadly to image other internal regions of the patient including, for example, the vasculature. The phrase "gastrointestinal region" or "gastrointestinal tract," as used herein, includes the region of a patient defined by the esophagus, stomach, small and large intestines and

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rectum. The present methods can also be used in connection with the delivery of a bioactive agent to an internal region of a patient.

As one skilled in the art would recognize, administration of the lipid and/or vesicle compositions of the present invention can be carried out in various fashions, 5 namely, parenterally, orally, or intraperitoneally. Parenteral administration, which is preferred, includes administration by the following routes: intravenous; intramuscular; interstitially; intra-arterially; subcutaneous; intraocular; intrasynovial; transepithelial, including transdermal; pulmonary via inhalation; ophthalmic; sublingual and buccal; topically, including ophthalmic; dermal; ocular; rectal; and nasal inhalation via 10 insufflation. Intravenous administration is preferred among the routes of parenteral administration. The useful dosage to be administered and the particular mode of administration will vary depending upon the age, weight and the particular mammal and region thereof to be scanned, and the particular contrast agent employed. Typically, dosage is initiated at lower levels and increased until the desired contrast enhancement is 15 achieved. Various combinations of the lipid compositions may be used to alter properties as desired, including viscosity, osmolarity or palatability. In carrying out the imaging methods of the present invention, the contrast medium can be used alone, or in combination with diagnostic, therapeutic or other agents. Such other agents include excipients such as flavoring or coloring materials. CT imaging techniques which are 20 employed are conventional and are described, for example, in *Computed Body Tomography*, Lee, J.K.T., Sagel, S.S., and Stanley, R.J., eds., 1983, Ravens Press, New York, N.Y., especially the first two chapters thereof entitled "*Physical Principles and Instrumentation*", Ter-Pogossian, M.M., and "*Techniques*", Aronberg, D.J., the disclosures of which are incorporated by reference herein in their entirety.

In the case of diagnostic applications, such as ultrasound and CT, energy, 25 such as ultrasonic energy, is applied to at least a portion of the patient to image the target tissue. A visible image of an internal region of the patient is then obtained, such that the presence or absence of diseased tissue can be ascertained. With respect to ultrasound, ultrasonic imaging techniques, including second harmonic imaging, and gated imaging, are 30 well known in the art, and are described, for example, in Uhlendorf, "Physics of Ultrasound Contrast Imaging: Scattering in the Linear Range", *IEEE Transactions on*

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Ultrasonics, Ferroelectrics, and Frequency Control, Vol. 14(1), pp. 70-79 (1994) and Sutherland, et al., "Color Doppler Myocardial Imaging: A New Technique for the Assessment of Myocardial Function", *Journal of the American Society of Echocardiography*, Vol. 7(5), pp. 441-458 (1994), the disclosures of which are hereby 5 incorporated herein by reference in their entirety.

Ultrasound can be used for both diagnostic and therapeutic purposes. In diagnostic ultrasound, ultrasound waves or a train of pulses of ultrasound may be applied with a transducer. The ultrasound is generally pulsed rather than continuous, although it may be continuous, if desired. Thus, diagnostic ultrasound generally involves the 10 application of a pulse of echoes, after which, during a listening period, the ultrasound transducer receives reflected signals. Harmonics, ultraharmonics or subharmonics may be used. The second harmonic mode may be beneficially employed, in which the $2x$ frequency is received, where x is the incidental frequency. This may serve to decrease the signal from the background material and enhance the signal from the transducer using the 15 targeted contrast media of the present invention which may be targeted to the desired site, for example, blood clots. Other harmonics signals, such as odd harmonics signals, for example, $3x$ or $5x$, would be similarly received using this method. Subharmonic signals, for example, $x/2$ and $x/3$, may also be received and processed so as to form an image.

In addition to the pulsed method, continuous wave ultrasound, for example, 20 Power Doppler, may be applied. This may be particularly useful where rigid vesicles, for example, vesicles formulated from polymethyl methacrylate, are employed. In this case, the relatively higher energy of the Power Doppler may be made to resonate the vesicles and thereby promote their rupture. This can create acoustic emissions which may be in the subharmonic or ultraharmonic range or, in some cases, in the same frequency as the 25 applied ultrasound. It is contemplated that there will be a spectrum of acoustic signatures released in this process and the transducer so employed may receive the acoustic emissions to detect, for example, the presence of a clot. In addition, the process of vesicle rupture may be employed to transfer kinetic energy to the surface, for example of a clot to promote clot lysis. Thus, therapeutic thrombolysis may be achieved during a combination of 30 diagnostic and therapeutic ultrasound. Spectral Doppler may also be employed. In general, the levels of energy from diagnostic ultrasound are insufficient to promote the

rupture of vesicles and to facilitate release and cellular uptake of the bioactive agents. As noted above, diagnostic ultrasound may involve the application of one or more pulses of sound. Pauses between pulses permits the reflected sonic signals to be received and analyzed. The limited number of pulses used in diagnostic ultrasound limits the effective 5 energy which is delivered to the tissue that is being studied.

Higher energy ultrasound, for example, ultrasound which is generated by therapeutic ultrasound equipment, is generally capable of causing rupture of the vesicle species. In general, devices for therapeutic ultrasound employ from about 10 to about 100% duty cycles, depending on the area of tissue to be treated with the ultrasound. Areas 10 of the body which are generally characterized by larger amounts of muscle mass, for example, backs and thighs, as well as highly vascularized tissues, such as heart tissue, may require a larger duty cycle, for example, up to about 100%.

In therapeutic ultrasound, continuous wave ultrasound is used to deliver higher energy levels. For the rupture of vesicles, continuous wave ultrasound is preferred, 15 although the sound energy may be pulsed also. If pulsed sound energy is used, the sound will generally be pulsed in echo train lengths of from about 8 to about 20 or more pulses at a time. Preferably, the echo train lengths are about 20 pulses at a time. In addition, the frequency of the sound used may vary from about 0.025 to about 100 megahertz (MHz). In general, frequency for therapeutic ultrasound preferably ranges between about 0.75 and 20 about 3 MHz, with from about 1 and about 2 MHz being more preferred. In addition, energy levels may vary from about 0.5 Watt (W) per square centimeter (cm^2) to about 5.0 W/cm^2 , with energy levels of from about 0.5 to about 2.5 W/cm^2 being preferred. Energy levels for therapeutic ultrasound involving hyperthermia are generally from about 5 W/cm^2 to about 50 W/cm^2 . For very small vesicles, for example, vesicles having a diameter of 25 less than about 0.5 μm , higher frequencies of sound are generally preferred. This is because smaller vesicles are capable of absorbing sonic energy more effectively at higher frequencies of sound. When very high frequencies are used, for example, greater than about 10 MHz, the sonic energy will generally penetrate fluids and tissues to a limited depth only. Thus, external application of the sonic energy may be suitable for skin and 30 other superficial tissues. However, it is generally necessary for deep structures to focus the ultrasonic energy so that it is preferentially directed within a focal zone. Alternatively,

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the ultrasonic energy may be applied via interstitial probes, intravascular ultrasound catheters or endoluminal catheters. Such probes or catheters may be used, for example, in the esophagus for the diagnosis and/or treatment of esophageal carcinoma. In addition to the therapeutic uses discussed above, the present compositions can be employed in connection with esophageal carcinoma or in the coronary arteries for the treatment of atherosclerosis, as well as the therapeutic uses described, for example, in U.S. Patent No. 5,149,319, the disclosures of which are hereby incorporated herein by reference, in their entirety.

A therapeutic ultrasound device may be used which employs two frequencies of ultrasound. The first frequency may be x , and the second frequency may be $2x$. In preferred form, the device would be designed such that the focal zones of the first and second frequencies converge to a single focal zone. The focal zone of the device may then be directed to the targeted compositions, for example, targeted vesicle compositions, within the targeted tissue. This ultrasound device may provide second harmonic therapy with simultaneous application of the x and $2x$ frequencies of ultrasound energy. It is contemplated that, in the case of ultrasound involving vesicles, this second harmonic therapy may provide improved rupturing of vesicles as compared to ultrasound energy involving a single frequency. Also, it is contemplated that the preferred frequency range may reside within the fundamental harmonic frequencies of the vesicles. Lower energy may also be used with this device. An ultrasound device which may be employed in connection with the aforementioned second harmonic therapy is described, for example, in Kawabata, K. et al., *Ultrasonics Sonochemistry*, Vol. 3, pp. 1-5 (1996), the disclosures of which are hereby incorporated herein by reference, in their entirety.

The concentration of lipid required to form a desired stabilized vesicle level will vary depending upon the type of lipid used, and may be readily determined by routine experimentation. For example, in preferred embodiments, the concentration of 1,2-dipalmitoylphosphatidylcholine (DPPC) used to form stabilized vesicles according to the methods of the present invention is about 0.1 mg/ml to about 30 mg/ml of saline solution, more preferably from about 0.5 mg/ml to about 20 mg/ml of saline solution, and most preferably from about 1 mg/ml to about 10 mg/ml of saline solution. The concentration of distearoylphosphatidylcholine (DSPC) used in preferred embodiments is about 0.1 mg/ml

to about 30 mg/ml of saline solution, more preferably from about 0.5 mg/ml to about 20 mg/ml of saline solution, and most preferably from about 1 mg/ml to about 10 mg/ml of saline solution. The amount of composition which is administered to a patient can vary. Typically, the IV dose may be less than about 10 mL for a 70 Kg patient, with lower doses being preferred.

In addition to the methods disclosed above, another embodiment of preparing a targeted contrast medium comprises combining at least one biocompatible lipid and a gaseous precursor; agitating until gas filled vesicles are formed; adding a targeting ligand to said gas filled vesicles such that the targeting ligand binds to said gas filled vesicle by a covalent bond or non-covalent bond; and agitating until a contrast agent comprising gas filled vesicles and a targeting ligand result. Rather than agitating until gas filled vesicles are formed before adding the targeting ligand, the gaseous precursor may remain a gaseous precursor until the time of use. That is, the gaseous precursor is used to prepare the contrast medium and the precursor is activated *in vivo*, by temperature for example.

Alternatively, a method of preparing a contrast medium targeted to endothelial cells may comprise combining at least one biocompatible lipid and a targeting ligand such that the targeting ligand binds to said lipid by a covalent bond or non-covalent bond, adding a gaseous precursor and agitating until a contrast medium comprising gas filled vesicles and a targeting ligand result. In addition, the gaseous precursor may be added and remain a gaseous precursor until the time of use. That is, the gaseous precursor is used to prepare the contrast medium having gaseous precursor filled vesicles and a targeting ligand which result for use *in vivo*.

Alternatively, the gaseous precursors may be utilized to create stable gas filled vesicles with targeting ligands which are pre-formed prior to use. In this embodiment, the gaseous precursor and targeting ligand are added to a container housing a suspending and/or stabilizing medium at a temperature below the liquid-gaseous phase transition temperature of the respective gaseous precursor. As the temperature is then exceeded, and an emulsion is formed between the gaseous precursor and liquid solution, the gaseous precursor undergoes transition from the liquid to the gaseous state. As a result of this heating and gas formation, the gas displaces the air in the head space above the

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liquid suspension so as to form gas filled lipid spheres which entraps the gas of the gaseous precursor, ambient gas for example, air, or coentrap gas state gaseous precursor and ambient air. This phase transition can be used for optimal mixing and stabilization of the contrast medium. For example, the gaseous precursor, perfluorobutane, can be entrapped
5 in the biocompatible lipid or other stabilizing compound, and as the temperature is raised, beyond 4° C (boiling point of perfluorobutane) stabilizing compound entrapped fluorobutane gas results. As an additional example, the gaseous precursor fluorobutane, can be suspended in an aqueous suspension containing emulsifying and stabilizing agents such as glycerol or propylene glycol and vortexed on a commercial vortexer. Vortexing is
10 commenced at a temperature low enough that the gaseous precursor is liquid and is continued as the temperature of the sample is raised past the phase transition temperature from the liquid to gaseous state. In so doing, the precursor converts to the gaseous state during the microemulsification process. In the presence of the appropriate stabilizing agents, surprisingly stable gas filled vesicles and targeting ligand result.

15 Accordingly, the gaseous precursors may be selected to form a gas filled vesicle *in vivo* or may be designed to produce the gas filled vesicle *in situ*, during the manufacturing process, on storage, or at some time prior to use.

It will be understood by one skilled in the art, once armed with the present disclosure, that the lipids, proteins, polymers and other stabilizing compounds used as
20 starting materials, or the vesicle final products, may be manipulated prior and subsequent to being subjected to the methods contemplated by the present invention. For example, the stabilizing compound such as a biocompatible lipid may be hydrated and then lyophilized, processed through freeze and thaw cycles, or simply hydrated. In preferred embodiments, the lipid is hydrated and then lyophilized, or hydrated, then processed through freeze and
25 thaw cycles and then lyophilized, prior to the formation of gaseous precursor filled vesicles.

According to the methods contemplated by the present invention, the presence of gas, such as and not limited to air, may also be provided by the local ambient atmosphere. The local ambient atmosphere may be the atmosphere within a sealed
30 container, or in an unsealed container, may be the external environment. Alternatively, for example, a gas may be injected into or otherwise added to the container having the

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aqueous lipid solution or into the aqueous lipid solution itself in order to provide a gas other than air. Gases that are not heavier than air may be added to a sealed container while gases heavier than air may be added to a sealed or an unsealed container. Accordingly, the present invention includes co-entrapment of air and/or other gases along with gaseous precursors.

As already described above in the section dealing with the stabilizing compound, the preferred methods contemplated by the present invention are carried out at a temperature below the gel state to liquid crystalline state phase transition temperature of the lipid employed. By "gel state to liquid crystalline state phase transition temperature", it is meant the temperature at which a lipid bilayer will convert from a gel state to a liquid crystalline state. See, for example, Chapman *et al.*, *J. Biol. Chem.* 1974, 249, 2512-2521.

Hence, the stabilized vesicle precursors described above, can be used in the same manner as the other stabilized vesicles used in the present invention, once activated by application to the tissues of a host, where such factors as temperature or pH may be used to cause generation of the gas. It is preferred that this embodiment is one wherein the gaseous precursors undergo phase transitions from liquid to gaseous states at near the normal body temperature of said host, and are thereby activated by the temperature of said host tissues so as to undergo transition to the gaseous phase therein. More preferably still, this method is one wherein the host tissue is human tissue having a normal temperature of about 37°C, and wherein the gaseous precursors undergo phase transitions from liquid to gaseous states near 37°C.

All of the above embodiments involving preparations of the stabilized gas filled vesicles used in the present invention, may be sterilized by autoclave or sterile filtration if these processes are performed before either the gas instillation step or prior to temperature mediated gas conversion of the temperature sensitive gaseous precursors within the suspension. Alternatively, one or more anti-bactericidal agents and/or preservatives may be included in the formulation of the contrast medium, such as sodium benzoate, all quaternary ammonium salts, sodium azide, methyl paraben, propyl paraben, sorbic acid, ascorbylpalmitate, butylated hydroxyanisole, butylated hydroxytoluene, chlorobutanol, dehydroacetic acid, ethylenediamine, monothioglycerol, potassium benzoate, potassium metabisulfite, potassium sorbate, sodium bisulfite, sulfur dioxide, and

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organic mercurial salts. Such sterilization, which may also be achieved by other conventional means, such as by irradiation, will be necessary where the stabilized microspheres are used for imaging under invasive circumstances, for example, intravascularly or intraperitoneally. The appropriate means of sterilization will be
5 apparent to the artisan instructed by the present description of the stabilized gas filled vesicles and their use. The contrast medium is generally stored as an aqueous suspension but in the case of dried vesicles or dried lipidic spheres the contrast medium may be stored as a dried powder ready to be reconstituted prior to use.

The novel compositions of the present invention, and especially the vesicle
10 compositions, are useful as contrast media in diagnostic imaging, and are also suitable for use in all areas where diagnostic imaging is employed. However, the stabilized vesicles are particularly useful for perfusion imaging.

Diagnostic imaging is a means to visualize internal body regions of a patient. Diagnostic imaging includes, for example, ultrasound (US), magnetic resonance
15 imaging (MRI), nuclear magnetic resonance (NMR), computed tomography (CT), electron spin resonance (ESR); nuclear medicine when the contrast medium includes radioactive material; and optical imaging, particularly with a fluorescent contrast medium. Diagnostic imaging also includes promoting the rupture of the vesicles via the methods of the present invention. For example, ultrasound may be used to visualize the vesicles and verify the
20 localization of the vesicles in certain tissue. In addition, ultrasound may be used to promote rupture of the vesicles once the vesicles reach the intended target, including tissue and/or receptor destination, thus releasing a bioactive agent and/or diagnostic agent.

In accordance with the present invention, there are provided methods of imaging a patient generally, and/or in specifically diagnosing the presence of diseased
25 tissue in a patient. The imaging process of the present invention may be carried out by administering a contrast medium of the invention to a patient, and then scanning the patient using, for example, ultrasound, computed tomography, and/or magnetic resonance imaging, to obtain visible images of an internal region of a patient and/or of any diseased tissue in that region. By region of a patient, it is meant the whole patient or a particular
30 area or portion of the patient. The contrast medium may be particularly useful in providing images of tissue, such as myocardial, endothelial, and/or epithelial tissue, as

well as the gastrointestinal and cardiovascular regions, but can also be employed more broadly, such as in imaging the vasculature or in other ways as will be readily apparent to those skilled in the art. Cardiovascular region, as that phrase is used herein, denotes the region of the patient defined by the heart and the vasculature leading directly to and from 5 the heart. The phrase vasculature, as used herein, denotes the blood vessels (arteries, veins, etc.) in the body or in an organ or part of the body. The patient can be any type of mammal, but most preferably is a human.

The present invention also provides a method of diagnosing the presence of diseased tissue. Diseased tissue includes, for example, endothelial tissue which results 10 from vasculature that supports diseased tissue. As a result, the localization and visualization of endothelial tissue to a region of a patient which under normal circumstances is not associated with endothelial tissue provides an indication of diseased tissue in the region.

In carrying out the magnetic resonance imaging method of the present 15 invention, the contrast medium can be used alone, or in combination with other diagnostic, therapeutic or other agents. Such other agents include excipients such as flavoring or coloring materials. The magnetic resonance imaging techniques which are employed are conventional and are described, for example, in D.M. Kean and M.A. Smith, *Magnetic Resonance Imaging: Principles and Applications*, (William and Wilkins, Baltimore 1986). 20 Contemplated MRI techniques include, but are not limited to, nuclear magnetic resonance (NMR) and electronic spin resonance (ESR). The preferred imaging modality is NMR.

Exemplary methods which may be employed in evaluating contrast agents of the present invention are discussed below with reference to the system illustrated in the drawings. Referring to the drawings, wherein like numerals refer to like elements, Figure 25 1 is a schematic representation of a system 10 for the *in vitro* evaluation of contrast agents in accordance with embodiments of the present invention. In the presently preferred embodiment, system 10 includes an apparatus 12 for evaluating the effectiveness of contrast agents in accordance with the present invention. The apparatus 12 includes a block 14, preferably composed of agar, such as 2% agar. Agar osc is commercially 30 available, for example, from Boehringer Mannheim (Indianapolis, IN), and may be block prepared using techniques well known to the ordinarily skilled artisan. The apparatus 12

further includes tubing 16 such as, for example, polyethylene tubing, which passes through the block 14. A string 18, such as a nylon string, is threaded through the tubing 16 to which a blood clot 20 is attached via an absorbent material 22, such as cotton piping material, that is fastened to the string 18 in a portion of the tubing 16 that passes through 5 the block 12. An ultrasound probe 24 is positioned proximate the block 14.

The contrast agents may be evaluated using the system 10 in Figure 10 using the following procedure. Liquid media, such as saline, may be pumped through the tubing 16 at a desired flow rate using a peristaltic pump (not shown). Contrast agents may be injected into the circulating solution upstream of the clot 20, and ultrasound signals 10 from the probe 24 may be directed at the region proximate the clot 20. Imaging may be performed continuously, and ultrasound intensity of the injected contrast agents may be measured at the region proximate the clot 20.

In accordance with another embodiment of the invention, as shown in Figure 7, targeted vesicle compositions which may be bound to a receptor, for example, 15 via a target-receptor interaction, preferably on a per vesicle basis, may create a much stronger acoustic response than non-targeted vesicle compositions (see also Figures 5 and 6). The novel methods described herein may be used for improving signal-to-noise for diagnostic and therapeutic applications, and may advantageously increase the sensitivity of ultrasound to vesicle compositions. In the case of thrombus detection, this is particularly 20 useful for detecting enhancement in old or echogenic thrombus. For other applications, the methods described herein may also be useful for detecting low concentrations of vesicles, for example, vesicles bound to endothelial targets, such as integrins associated with malignancy or inflammation in early or small lesions.

The methods of the invention may employ a transducer, preferably of 25 piezoelectric material, for example, lead zirconate, with a narrow emission band of frequencies. In particularly preferred embodiments, the bandwidth of the insonation frequency may range from about greater than 0 to about 100 kHz (and all combinations and subcombinations of ranges therein). Preferably, the bandwidth of the insonation frequency may be about 100 kHz or less, and more preferably about 50 kHz, and even 30 more preferably less than about 10 kHz. Also in particularly preferred embodiments, the bandwidth of the center insonation frequency may range from greater than about 0 to about

1000 Hz (and all combinations and subcombinations of ranges therein), with less than about 1000 Hz being preferred. The methods of the invention may employ a broad band receiver, preferably extending over a range of several megahertz, for example, from about 100 kHz to about 14 MHz, and all combinations and subcombinations of ranges therein. A 5 digital receiver filter gate may also be employed with multiple center frequencies. The center frequencies may be preferably arranged within the broadband receiver digitally adjusted with respect to the insonating frequency. Each filter gate may be centered with respect to the insonating frequency as shown in Figure 9. The filter gates and bandwidth windows may be selected digitally and digitally controlled. The filter may desirably reject 10 signals with center frequencies outside of the range. The filter thereby may receive the peak of each sub-harmonic and harmonic signal and suppress background noise.

In addition to the narrow excitation frequency, the methods of the invention may also involve novel pulsing and phase modulation. It has also been discovered that dual frequency insonation may further increase the signal. By first insonating at a lower 15 frequency, for example, about 1 MHz, and secondly insonating at a higher frequency, for example, about 3 MHz, the signal may be greatly amplified. In this respect, two or more frequencies may be selected, for example, a first frequency ranging from about 20 kHz to about 10 MHz (and all combinations and subcombinations of ranges therein) and a second frequency ranging from about 20 kHz to about 50 MHz (and all combinations and 20 subcombinations of ranges therein). Generally speaking, the second frequency of insonation may be at least about twice the first insonation frequency. The first insonation frequency, however, may be much lower than the second insonation frequency, for example, about 100 kHz may advantageously be employed with about 3 MHz as the second insonating frequency. In this regard, the first, low frequency insonation may be 25 administered as a pulse of sound, which may be a pulse train of up to a hundred pulses, but preferably may be administered as a shorter train of pulses of about 10 or less pulses. In particularly preferred embodiments, a single pulse of low frequency sound may be applied, which may be followed in rapid succession by pulses of higher frequency sound. For example, a single pulse of about 100 kHz sound may be applied and within about 40 30 milliseconds one or preferably several pulses of higher frequency ultrasound may then be applied. The result may be thought of as first stimulating the vesicle and then secondly

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sampling the vesicle. The first pulse may stimulate the vesicle into an oscillating maximally efficient reflector, while the second set of pulses may interrogate the vesicle. Summation of the several pulses given in rapid succession after the initial stimulation pulse may thereby maximize the signal-to-noise ratio.

- 5 In a further embodiment of this invention, the phase and/or time delay of the ultrasound pulses may also be varied. The first and second pulses, which may be of different amplitude and frequency or the same amplitude and frequency, may be phase modulated. Phase and/or delay variation enables improved background signal suppression. For therapeutic purposes, the phase and/or time delay of the second pulse may be selected
- 10 to maximize vesicle oscillation and collapse. Pulse one in this situation may be a pulse train, with a pulse duration preferably ranging from about 10 microseconds to about 10 seconds (and all combinations and subcombinations of ranges therein), more preferably from about 1 millisecond to about 2 seconds. The peak acoustic pressure for pulse one may range from about 100 pascals to about 10 megapascals (and all combinations and
- 15 subcombinations of ranges therein), preferably about 1 kilopascal to about 5 megapascals, and more preferably about 10 kilopascals to about 5 megapascals.

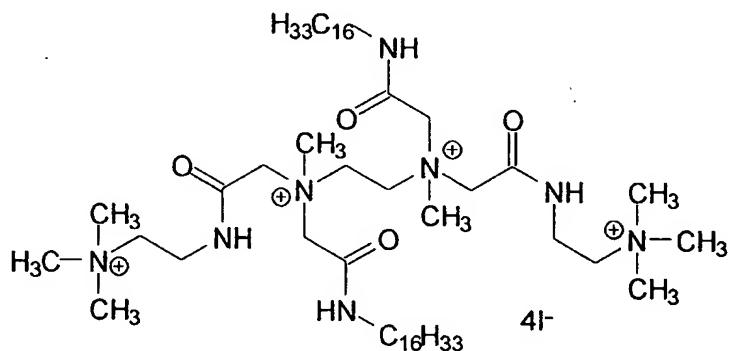
- The invention is further demonstrated in the following examples. Examples 1 to 8, 13 to 15, 20 to 22, 27, 28, 42, 43 to 45 and 47, 48, 60 and 61 are actual examples and Examples 9 to 12, 16 to 19, 23 to 26, 29 to 41 and 49 to 59 are prophetic examples.
- 20 Example 46 is both actual (in part) and prophetic (in part). The examples are for purposes of illustration and are not intended to limit the scope of the present invention.

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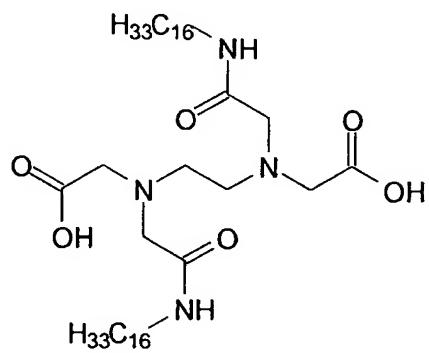
EXAMPLES

Example 1

- This example is directed to the preparation of N,N'-bis(hexadecylaminocarbonylmethylene)-(β-N,N,N-trimethylammonium 5 ethylaminocarbonylmethylene)-N,N'-dimethyl-N,N'-ethylenediamine tetraiodide (EDTA-HA-TMA tetraiodide), which has the following formula.



- A. Preparation of N,N'-bis-(hexadecylaminocarbonylmethylene)-ethylenediamine-N,N'-diacetic acid (EDTA-HA).



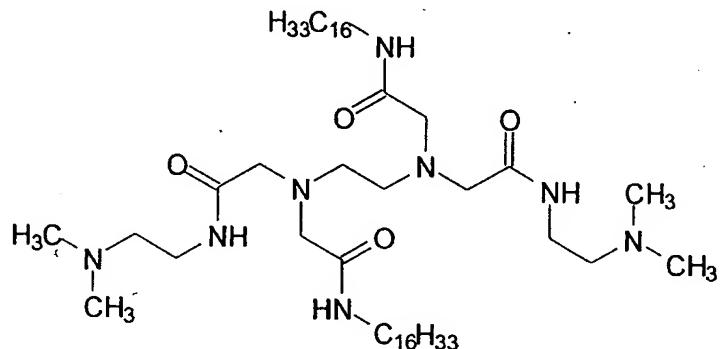
- Ethylenediaminetetraacetic acid dianhydride (2.56 g, 0.01 mole) in dry methanol (30 mL) and hexadecylamine (4.82 g, 0.02 mole) in dry methanol (60 mL) were combined and stirred at 50°C for 6 hours. The resulting white solid was isolated by

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filtration and dried at room temperature under vacuum to yield 3.43 g (64%) of EDTA-HA.

IR: 3320 cm⁻¹ for OH, 1670 cm⁻¹ for C=O (carbonyl).

- B. Preparation of N,N'-bis-(hexadecylaminocarbonylmethylene)-N,N'-β-5 N,N-dimethylamino-ethylaminocarbonylmethylene)ethylenediamine
(EDTA-HA-DMA).



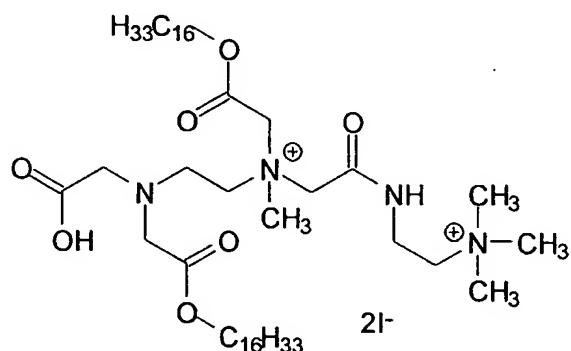
- To a cooled (5°C) solution of EDTA-HA from Step A (3.69 g, 0.005 mole), N,N-dimethylethylenediamine (0.88 g, 0.01 mole) and CHCl₃ (100 mL) was added dropwise a solution of DCC (2.227 g, 0.011 mole) in CHCl₃ (20 mL). The resulting 10 emulsion was stirred at room temperature for about 24 hours and filtered. The filtrate was washed with 0.5% acetic acid (100 mL) to decompose any excess DCC. A white milky solution was observed which separated into two layers. The lower organic layer was dried overnight (Na₂SO₄) and concentrated *in vacuo* to provide 3.81 g of EDTA-HA-DMA as a soft solid.
- 15 IR: 3280 cm⁻¹, 2900 cm⁻¹, 1640 cm⁻¹, 1530 cm⁻¹
- C. Preparation of EDTA-HA-TMA tetraiodide.
- EDTA-HA-DMA from Step B (4.22 g, 4.77 mmole), iodomethane (3.41 g, 24 mmole) and ethanol (30 mL) were combined and refluxed for 2 hours. The reaction mixture was concentrated and the residue was lyophilized overnight. 4.98 g of the title 20 product (EDTA-HA-TMA tetraiodide) was obtained as a yellow solid.

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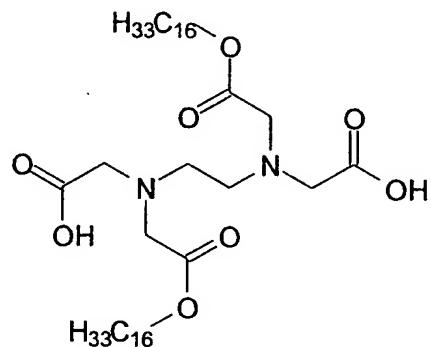
IR: 3260 cm⁻¹, 1650 cm⁻¹.

Example 2

This example is directed to the preparation of N,N'-bis(hexadecyloxycarbonylmethylene)-N-(β-N,N,N-trimethylammoniummethyl-5 aminocarbonylmethylene)-N-methyl-N'-(carboxymethylene)ethylenediamine diiodide (EDTA-HAL-DMA diiodide), which has the following formula.



A. Preparation of N,N'-bis-(hexadecyloxycarbonylmethylene)-ethylenediamine-N,N'-diacetic acid (EDTA-HAL).

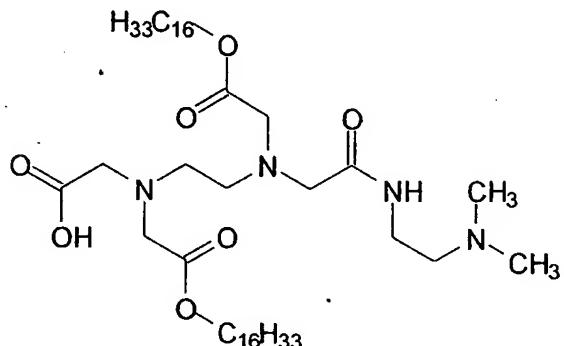


Hexadecyl alcohol (4.84 g, 0.02 mole), dry dimethylformamide (20 mL),
10 dry triethylamine (3.3 g) and ethylenediaminetetraacetic acid dianhydride (2.56 g, 0.01 mole) were combined and stirred at 50°C for 2 hrs. The reaction mixture was poured into

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cold water (200 mL) and the resulting aqueous mixture was acidified with HCl. The resulting white precipitate was isolated by filtration and the filter cake was washed with water. Recrystallization of the white solid from ethanol yielded 7 g of EDTA-HAL. m.p. 103°C.

5 B. Preparation of N,N'-bis(hexadecyloxycarbonylmethylene)-N-(β-N-dimethylaminoethylaminocarbonyl)-N'-acetic acid (EDTA-HAL-DMA).



To a cooled (5°C) solution of EDTA-HAL from Step A (3.70 g, 0.005 mole), N,N-dimethylethylenediamine (1.598 g, 0.0175 mole) and CHCl₃ (100 mL) was added dropwise a solution of (DCC) (3.60 g, 0.0175 mole) in CHCl₃ (20 mL). A precipitate formed, and the reaction mixture was stirred at room temperature for about 24 hours. The reaction mixture was filtered and the filtrate was washed with 0.5% acetic acid (100 mL) to decompose any excess DCC. A white milky solution formed which separated into two layers. The bottom organic layer was dried and concentrated *in vacuo* to yield 3.85 g of EDTA-HAL-DMA as a viscous liquid.

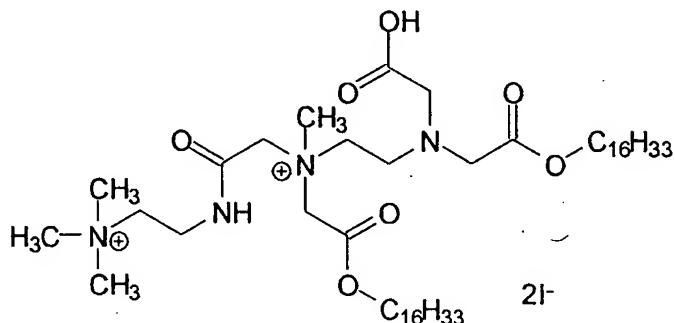
15 C. Preparation of EDTA-HAL-DMA diiodide.

EDTA-HAL-DMA from Step B (2.36 g, 0.0027 mole), iodomethane (3.81 g) and ethanol (50 mL) were combined and refluxed for 2 hrs. The solution was concentrated *in vacuo* and the resulting residue was lyophilized overnight. 2.96 g of the title compound (EDTA-HAL-DMA diiodide) was obtained as a yellowish solid.

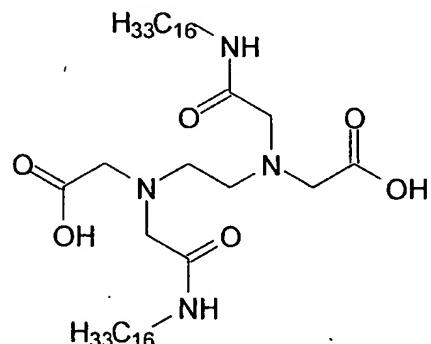
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Example 3

This example is directed to the preparation of N,N'-bis(hexadecyloxycarbonylmethylene)-N-(β -N,N,N-trimethylammoniummethylaminocarbonylmethylene)-N-methyl-N'-(carboxymethylene)ethylenediamine diiodide
 5 (EDTA-HA-DMA diiodide), which has the following formula.



A. Preparation of N,N'-bis(hexadecylaminocarbonylmethylene)-ethylenediamine-N,N'-diacetic acid (EDTA-HA).

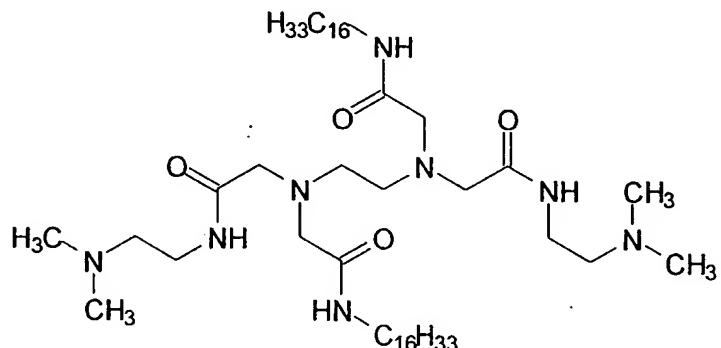


10 Hexadecylamine (4.82 g, 0.02 mole) in dry methanol (60 mL) was added to a suspension of ethylenediaminetetraacetic acid dianhydride (2.56 g, 0.01 mole) in dry methanol (30 mL). The mixture was stirred at 50°C for 6 hours. The resulting white solid precipitate was isolated by filtration and dried under vacuum at room temperature to yield 3.43 g (64%) of EDTA-HA. m.p. 156-158° C.

15 IR: 3320 cm⁻¹ for OH; 1670cm⁻¹ for -C(=O)-.

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B. Preparation of N,N'-bis(hexadecylaminocarbonylmethylene)-N,N'-bis(β -N,N-dimethylaminoethylaminocarbonylmethylene)ethylenediamine (EDTA-HA-DMA).



- To a cooled (5°C) solution of EDTA-HA from Step A (3.69 g, 0.005 mole), N,N-
- 5 dimethylethylenediamine (0.88 g, 0.01 mole) and CHCl₃ (100 mL) was added dropwise a solution of 1,3-dicyclohexylcarbodiimide (DCC) (2.227 g, 0.011 mole) in CHCl₃ (20 mL). A precipitate was observed and the reaction mixture was stirred at room temperature for about 24 hours. The reaction mixture was filtered and the filtrate was washed with 0.5% acetic acid (100 mL) to decompose any excess DCC. A white milky solution was formed
- 10 which separated into two layers. The bottom organic layer was dried (Na₂SO₄) and concentrated *in vacuo* to yield 3.81 g of EDTA-HA-DMA as a soft solid.

IR: 3280 cm⁻¹; 2900 cm⁻¹; 1640 cm⁻¹; 1530 cm⁻¹.

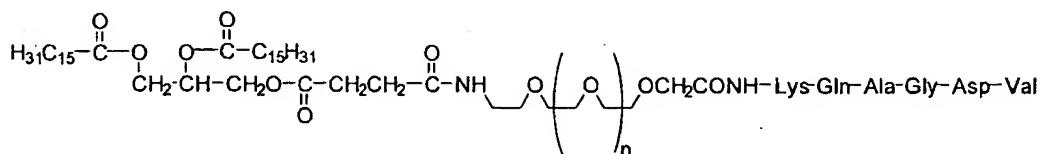
C. Preparation of EDTA-HA-DMA diiodide.

- A solution of EDTA-HA-DMA from Step B (4.2 g, 4.77 mmole),
- 15 iodomethane (3.41 g, 24 mmole) and ethanol (30 mL) was refluxed for 2 hours. The ethanolic solution was concentrated *in vacuo* and the resulting residue was lyophilized overnight. 3.98 g of the title compound (EDTA-LA-DMA diiodide) was obtained as a yellow solid.

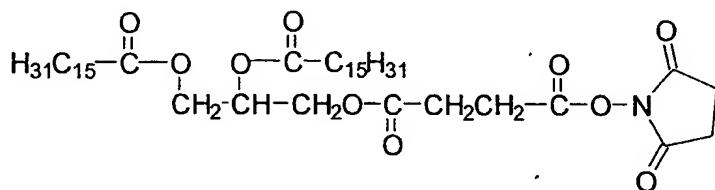
IR: 3260 cm⁻¹; 1650 cm⁻¹.

Example 4

This example is directed to the preparation of DPGS-PEG-Lys-Gln-Ala-Gly-Asp-Val which has the following formula.

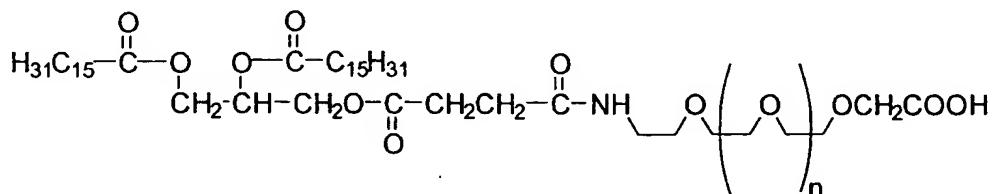


- 5 A. Preparation of N-(1,2-dipalmitoyl-sn-glycero-3-succinyl)-succinimide
(N-DPGS-succinimide).



- To a cooled (0 to 5 °C) solution of DCC (20.6 mg) and acetonitrile (10 mL) was added dropwise a solution of 1,2-dipalmitoyl-sn-glycero-3-succinate (Avanti Polar 10 Lipids, Alabaster, AL) (66.8 mg), N-hydroxysuccinimide (11.5 mg), dimethylaminopyridine (DMAP) (2 mg) and acetonitrile (40 mL). The reaction mixture was stirred for 5 hours and the resulting solid was removed by filtration. The filtrate was concentrated *in vacuo* to provide 78 mg of N-DPGS-succinimide as a white product.

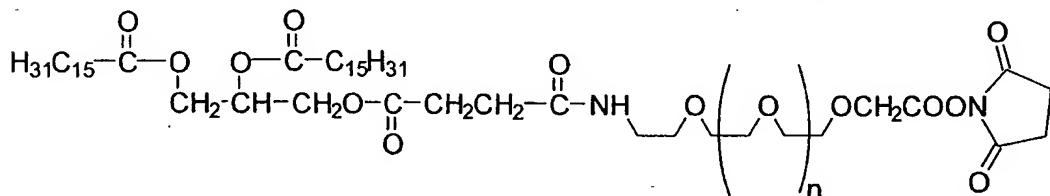
- B. Preparation of 3- ω -carboxy-polychthleneglycoliminosuccinate-1,2-dipamitoyl-sn-glycerol (DPGS- ω -carboxy-PEG).



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To a cooled (0 to 5°C) solution of ω -amino- ω' -carboxy-polyethyleneglycol (Shearwater Polymers, Huntsville, AL) (0.3 g) and triethylamine (40 mg) in CHCl₃ (20 mL) was added dropwise a solution of N-DPGS-succinimide from Step A (78 mg) in CHCl₃ (10 mL). The resulting solution was stirred for about 5 hours at 10°C and allowed 5 to stand overnight. The reaction mixture was poured into ice water and neutralized with 10% HCl to a pH of less than 3. The organic layer was isolated, washed with water and dried (NaSO₄). Filtration and concentration *in vacuo* provided 0.34 g of DPGS- ω -carboxy-PEG as a white solid.

C. Preparation of 3-succinamoyloxycarbonyl-polyethyleneglycol-imino-10 succinate-1,2-dipalmitoyl-sn-glycerol (DPGS- ω -carboxy-PEG-succinimide).



To a cooled (0 to 5°C) solution of DCC (12 mg) in acetonitrile (10 mL) was added a solution of DPGS- ω -carboxy-PEG from Step B (200 mg), N-hydroxysuccinimide (6 mg) and dimethylaminopyridine (2 mg). The resulting reaction mixture was stirred for 5 hours. The white solid which formed was removed from the 15 reaction mixture by filtration, and the filtrate was concentrated *in vacuo*. DPGS- ω -carboxy-PEG-succinimide was obtained as a white solid (200 mg).

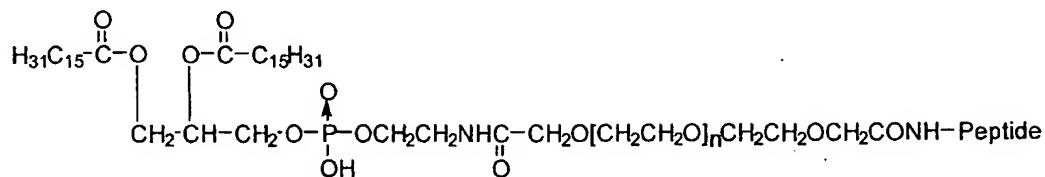
D. Preparation of DPGS-PEG-Lys-Gln-Ala-Gly-Asp-Val conjugate.

To a cooled (0 to 5°C) solution of the peptide Lys-Gln-Ala-Gly-Asp-Val (5 mg) in a buffer solution at pH 8.5 (20 mL) was added dropwise a solution of DPGS- ω -carboxy-PEG-succinimide from Step C (40 mg) in acetonitrile (0.1 mL). The resulting 20 reaction mixture was stirred at room temperature for about 48 hours. The reaction mixture was concentrated *in vacuo* and the mineral salt was dialyzed through a membrane having a molecular weight cutoff of about 1,000 and dried on a lyophilizer. The title compound (DPGS-PEG-Lys-Gln-Ala-Gly-Asp-Val) was obtained as a white solid (24 mg).

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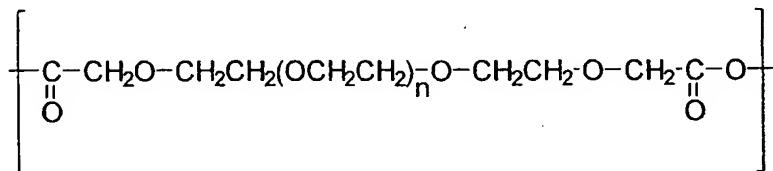
Example 5

This example is directed to the preparation of DPPE-PEG-Lys-Gln-Ala-Gly-Asp-Val), which has the following formula.



where "Peptide" is —Lys—Gln—Ala—Gly—Asp—Val

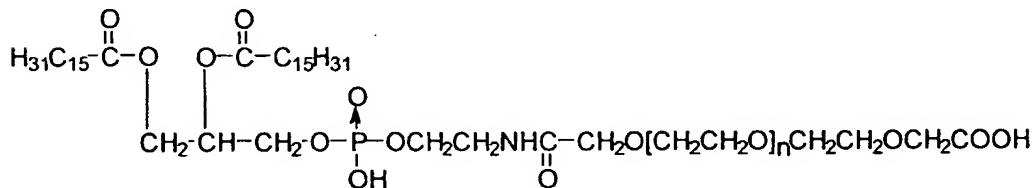
A. Preparation of ω,ω' -dimethylenecarboxy-polyethylene glycol anhydride.



5 To a cooled (0 to 5 °C) solution of chlorosulfonyl isocyanate (14.2 mg) in CHCl_3 (5 mL) was added a solution of ω,ω' -dimethylenecarboxy-polyethylene glycol (0.34 g) and triethylamine (20 mg) in CHCl_3 (20 mL). The reaction mixture was stirred overnight and poured into ice water. The organic layer was isolated and dried (NaSO_4). Filtration and concentration of the organic layer *in vacuo* yielded the title anhydride compound as a white solid (0.2 g).

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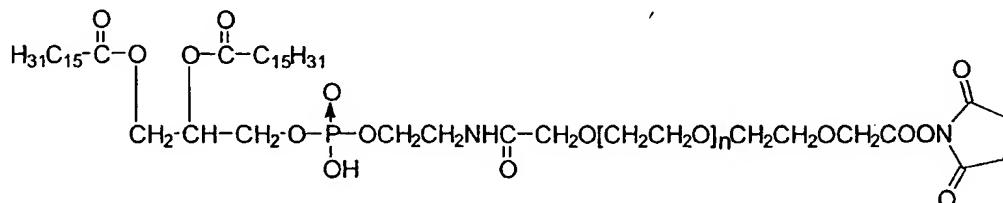
B. Preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N--carbonylmethylene- ω' -carboxy-polyethylene glycol (DPPE- ω -carboxy-PEG).



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To a cooled (0 to 10°C) solution of the anhydride compound from Step A (0.3 g) in CH₂Cl₂ (10 mL) was added a solution of DPPE (0.07 g) and triethylamine (0.05 g) in CH₂Cl₂ (15 mL). The resulting reaction mixture was stirred overnight, poured into ice water and neutralized with 10% HCl to a pH of less than 3. The organic layer was isolated and dried (NaSO₄). Filtration and concentration of the organic layer *in vacuo* provided 0.45 g of DPPE- ω -carboxy-PEG as a dark white solid.

C. Preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N-carbonylmethylene-polyethylene glycol-succinimide (DPPE- ω -carboxy-PEG-succinimide).



To a cooled (0 to 5°C) solution of DCC (3 mg) in acetonitrile (2 mL) was added a solution of DPPE- ω -carboxy-PEG from Step B (60 mg), N-hydroxy-succinimide (1.8 mg) and dimethylaminopyridine (0.2 mg) in acetonitrile (6 mL). The resulting mixture was stirred for 3 hours at 0 to 5°C and then overnight at room temperature. The solid which formed was removed by filtration and the filtrate was concentrated *in vacuo* to provide 60 mg of DPPE- ω -carboxy-PEG-succinimide.

D. Preparation of DPPE-PEG-Lys-Gln-Ala-Gly-Asp-Val conjugate.

To a cooled (0 to 5°C) solution of Lys-Gln-Ala-Gly-Asp-Val (5 mg) in a buffer solution at pH 8.5 was added dropwise DPPE- ω -carboxy-PEG-succinimide (40 mg) in acetonitrile (10 mL). The resulting mixture was stirred at room temperature for about 48 hours. The acetonitrile was removed *in vacuo*, and the mineral salt was dialyzed out through a membrane having a molecular weight cutoff of 1000. Lyophilization afforded 35 mg of the title compound (DPPE-PEG-Lys-Gln-Ala-Gly-Asp-Val) as a white solid.

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Example 6

To a solution of saline, propylene glycol and glycerol (8:1:1) were added DPPC, DPPE-PEG5000 and DPPA in a molar ratio of 82:8:10. The resulting mixture was heated to about 45°C and filtered (0.22 µm). The filtered mixture was placed in a vial and
5 allowed to cool to room temperature. The vial was placed under vacuum to evacuate any gas, after which the vial was pressurized with PFP. The vial was then sealed, placed on a shaker and agitated at room temperature to provide a solution of PFP-filled vesicles having a mean diameter of about 2.5 µm.

Example 7

10 This example is directed to the preparation of targeted vesicles within the scope of the present invention.

GPIIbIIIa binding peptide (Integrated Biomolecule Corporation, Tucson, AZ) was covalently bonded to DPPE-PEG 3400 utilizing the procedure described above in Example 5. This peptide conjugate was then combined with a dried lipid mixture of DPPC
15 (82 mole %), DPPE-PEG5000 (8 mole %) and DPPA (8 mole %). This mixture was hydrated and lyophilized on a Labconco Lyph-Lock 12 lyophilizer (Kansas City, MO). The lyophilized material was resuspended in 8:1:1 normal saline:propylene glycol:glycerol at a concentration of 1 mg/mL. Aliquots of this mixture were placed into 2 mL Wheaton vials (Millville, NJ), capped and the headspace replaced with perfluorobutane gas (Flura,
20 Newport, TN). The vials were agitated to provide a vesicle composition targeted to the GPIIbIIIa receptor.

Example 8

This example includes a description of peptide binding experiments utilizing the vesicle compositions prepared in Examples 6 and 7.

25 Unheparinized human blood was placed in Vacutainer tubes (Becton Dickinson, Rutherford, NJ) and allowed to clot. The clot was collected by centrifugation in a Beckman TJ-6 centrifuge (Palo Alto, CA). The clot was then placed on positively charged microscope slides (Fisher Scientific, Pittsburgh, PA). Nine slides were coated with clotted blood. Six uncoated slides were also used as control studies. Binding studies

were then conducted by applying the vesicle compositions from Examples 6 and 7 (200 µL) to the aforementioned slides as follows: (A) blood clot alone (control); (B) vesicle composition from Example 6 without blood clot; (C) vesicle composition from Example 6 with blood clot; (D) vesicle composition from Example 7 without blood clot; and (E)

5 vesicle composition from Example 3 with blood clot. The slides were incubated for 20 minutes and then unbound material was washed off using phosphate buffered saline. The slides were then observed using a Nikon Diaphot (Tokyo, Japan) microscope. The results are set forth in the following table.

TABLE 3

	<u>Binding Study</u>	<u>Binding</u>
	(A)	no
	(B)	no
	(C)	no
	(D)	no
15	(E)	yes

As can be seen from the above table, binding was observed only with the vesicle composition prepared in Example 7 which contains GPIIbIIIa targeting peptide.

Example 9

This example is directed to the preparation of vesicles based on human serum albumin that bear targeting ligands directed to the GPIIbIIIa receptor.

20 Into a vessel will be introduced a suspension of 5% human serum albumin. The suspension will be deaerated under continuous vacuum and a headspace of perfluoropropane gas will be introduced, as described in Published International Application WO 95/29705, the disclosures of which are hereby incorporated by reference, in their entirety. The resulting gas-filled albumin vesicles will be separated from any free albumin by repeated washing with normal saline. The gas-filled vesicles will be resuspended in a mixture of normal saline:propylene glycol:glycerol (6:2:2, v:v:v) and the suspension will be mixed gently. To this suspension will be added 1% glutaraldehyde and

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1% by weight of the peptide Lys-Gln-Ala-Gly-Asp-Val resulting in crosslinked perfluoropropane vesicles bearing GPIIbIIIa binding peptide.

Example 10

This example is directed to the preparation of vesicles stabilized by 5 polymerizable acryloyl/styryl lipid analogs binding targeting ligands directed to the GPIIbIIIa receptor.

12-Hydroxydodecanoic acid will be esterified with methacryloyl chloride and the ester will be converted to the anhydride to provide 12-(methacryloyloxy)dodecanoic anhydride. L-alpha-Glycerophosphocholine, derived from egg lecithin, 10 will be acylated with the 12-(methacryloyloxy)dodecanoic anhydride to afford bis[12-(methacryloyloxy)dodecanoyl]-L-alpha-phosphatidylcholine (1). Compound (1) will be hydrolyzed enzymatically with phospholipase A₂, derived from crude rattle snake venom (*Crotalus adamanteus*), followed by acylation with palmitoyl anhydride to provide 1-[2-(methacryloyloxy)dodecanoyl]-2-palmitoyl-L-alpha-phosphatidylcholine (2). Similar 15 methods will be employed to convert dipalmitoyl-L-alpha-phosphatidylcholine into 1-palmitoyl-2-[12-(methacryloyloxy)dodecanoyl]-L-alpha-phosphatidylcholine (3).

To a solution in normal saline of DPPE-PEG 5000 (1.8 mg/mL) and 10% by weight DPPE-PEG-5000 labelled with GPIIbIIIa binding peptide (Lys-Gln-Ala-Gly-20 Asp-Val), which will be prepared using the procedure described above in Example 5, will be added compound (3) prepared above. An aliquot of this solution will be placed in a sterile 3 mL vial. The headspace of the vial will be evacuated and a mixture of nitrogen and perfluoroethane gas (20:80, v/v) will be filled into the headspace of the vial to ambient pressure. The vial will be shaken on an ESPE Capmix (Seefeld, Oberay Germany) at room 25 temperature for about 1 minute. The vesicle composition will be irradiated (254 nm) to provide polymerized gas-filled vesicles bearing GPIIbIIIa binding peptide. The mean diameter of the vesicles will be about 3 μm .

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Example 11

This example is directed to the preparation of synthetic polybis(carboxylatophenoxy)phosphazene) (PCPP) gas-filled vesicles targeted to the GPIIbIIIa receptor.

5 PCPP-PEG2000-Lys-Gln-Ala-Gly-Asp-Val will be prepared utilizing the procedure described above in Example 5. PCPP vesicles will then be prepared as described as in U.S. Patent No. 5,487,390, the disclosures of which are hereby incorporated by reference herein, in their entirety. The PCPP vesicles (100 mg) will be added to 1 mL of a Na₂CO₃ solution (30 mg/mL) and will be dissolved by stirring
10 overnight at room temperature. The solution will be diluted with phosphate-buffered saline (pH 7.4) to provide a final polymer concentration of 2.5% (w/v) and a solution pH of 7.4. The pH will be adjusted, as needed, with 1N HCl.

A solution of PCPP (2.5% w/v) containing 0.2% Tween 20 will be mixed with perfluoropropane gas in a colloid mill to produce a gassed PCPP solution that is
15 stable for several hours. Ten mole % of the PCPP utilized in the solution will be PCPP-PEG2000-Lys-Gln-Ala-Gly-Asp-Val. The gassed solution will be extruded from a syringe pump at 150 µL/min into an air-atomizing device under an atmosphere of perfluoropropane gas and sprayed into a pan containing 250 mL of a 7.5% CaCl₂ solution containing 0.5% Tween 20. Upon contact with the CaCl₂ solution, PCPP will be
20 crosslinked by the divalent calcium ions to produce a relatively homogeneous population of spherical gel vesicles, targeted to the GPIIbIIIa and filled with perfluoropropane. The presence of entrapped perfluoropropane will be demonstrated by observation of the vesicles using an inverted microscope.

Example 12

25 This example is directed to the preparation of a vesicles targeted to the GPIIbIIIa receptor formulated form a fluorinated lipid.

The amino group in α-amino, ω-carboxy-PEG3400 (Shearwater Polymer, Huntsville, AL) will be protected by dissolving one equivalent of the α-amino, ω-carboxy-PEG3400 in a solution of water:dioxane (1: 1, v:v). To this stirring solution will be added
30 one molar equivalent each of t-Boc-anhydride (Bachem, Gardena, CA) and triethylamine

(Aldrich Chemical, Milwaukee, WI). The resulting solution will be stirred overnight and concentrated *in vacuo* to remove the volatile dioxane. To concentrated material will be added ten volume equivalents, relative to water, of ethyl acetate and the resulting mixture will be cooled with an ice bath. The pH of the mixture will be adjusted to pH 2 with 5 aqueous 2N sulfuric acid, and the lower aqueous layer will be removed using a separatory funnel. The ethyl acetate layer will be washed with saturated brine, dried ($MgSO_4$), filtered, and concentrated *in vacuo* to afford a syrup.

The t-BOC- α -amino, ω -carboxyl PEG3400, one molar equivalent of diisopropylcarbodiimide (DIC) (Sigma Chemical Co., St. Louis, MO) and dry DMF will 10 be combined. The solution will be stirred and one equivalent each of diisopropylethylamine (DIEA) (Sigma Chemical Co., St. Louis, MO), hydroxybenzotriazole (HOBT) and 1,2-di-(15,16,17,18-nonafluoro)stearoyl-phosphatidylethanolamine (fluoro-DSPE) will be added. The resulting solution will be stirred for 2 hours and concentrated *in vacuo*. The fluoro-DSPE-t-BOC- α -amino, ω -amido 15 PEG3400 will be suspended in a solution of trifluoroacetic acid and CH_2Cl_2 (4:6, v:v), followed by stirring an additional 20 minutes. The solution will then be neutralized with DIEA and concentrated *in vacuo*. The product will be resuspended in CH_2Cl_2 and aqueous HCl will be added to a pH of about 2. The aqueous layer will be separated and the pH readjusted to pH 1.0 with 1.0% NaOH. To the aqueous mixture will be added ethyl acetate 20 which will be separated, washed with saturated brine, and dried ($MgSO_4$). Filtration and concentration *in vacuo* will provide the fluoro-DSPE α -amino, ω -amido PEG3400 as a yellow oil.

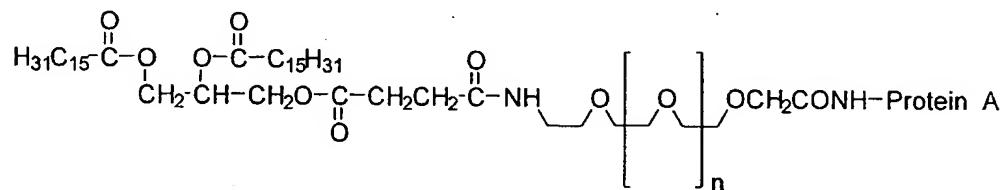
The yellow oil obtained above, one equivalent of N-bromosuccinimide (NBS), (Sigma Chemical, St. Louis, MO) and DMF will be combined. The resulting 25 mixture will be stirred for 3 hours and concentrated *in vacuo* overnight. The concentrated material will be suspended in phosphate-buffered saline at a pH of 7. The peptide Arg-Gly-Asp-Scr (RGDS), which is targeted to the GPIIbIIIa receptor, will be added to the suspension. After stirring overnight, the reaction mixture will be concentrated with an Amicon filter concentrator (Amicon, Beverly, MA) to a volume of ~300 mL. The 30 concentrated solution will be purified by size exclusion chromatography to yield the fluoro-DSPE- α -amino, ω -amido PEG3400-Arg-Gly-Asp-Scr. This product will be added

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to a mixture of DPPC and DPPA to provide a ratio of DPPC:fluoro-DSPE:DPPA of 40:54:6, w:w:w. This lipid mixture will be added to a diluent comprising normal saline:glycerol:propylene glycol (8:1:1, v:v:v) to provide a final lipid concentration of 1 mg/mL of diluent. An aliquot of this solution will be introduced into a 2 mL vial

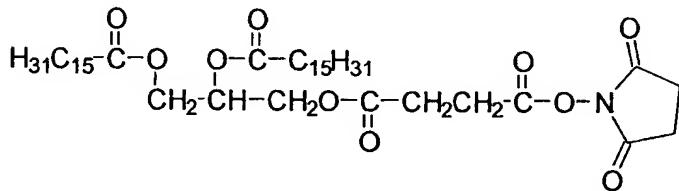
- 5 (Wheaton Industries, Chicago, IL) and the headspace will be filled with perfluoropropane. The vial will be agitated on an ESPE Capmix (ESPE, Seefeld, Germany) at 4300 rpm for 1 minute to yield the fluorinated vesicle targeted to the GPIIbIIIa receptor.

Example 13



- This example is directed to the preparation of N-(1,2-dipalmitoyl-sn-glycero-3-succinyl)-PEG-Protein A conjugate, which has the following formula.

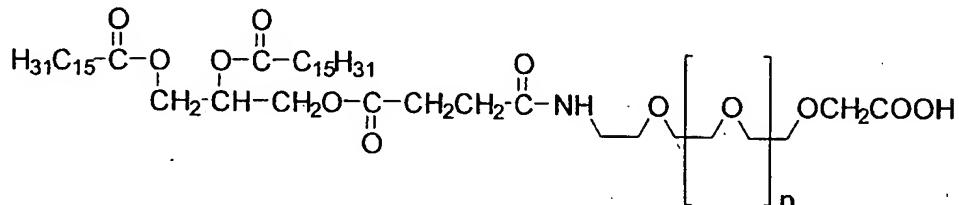
A. Preparation of N-DPGS-succinimide.



- To a cooled (0 to 5°C) solution of 1,2-dipalmitoyl-sn-glycero-3-succinate (66.8 mg), N-hydroxy-succinimide (11.5 mg), DMAP (2 mg) and acetonitrile (40 mL) in a 100 mL round bottom flask was added dropwise a solution of DCC (20.6 mg) in 15 acetonitrile (10 mL). The resulting mixture was stirred for 5 hours. The solid material which formed during the reaction (dicyclohexylurea) was removed by filtration and the filtrate was concentrated *in vacuo* to yield 78 mg of N-DPGS-succinimide as a white product.

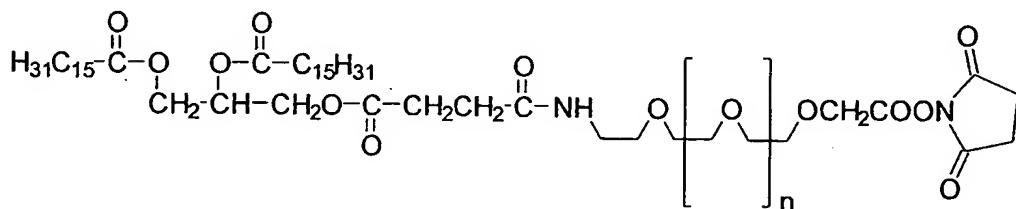
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B. Preparation of 3- ω -carboxy-polyethyleneglycol-imino-succinat-1,2-dipalmitoyl-sn-glycerol (DPGS- ω -carboxy-PEG).



To a cooled (0 to 5°C) solution of N-DPGS-succinimide from Step A (78 mg) and CHCl₃ (10 mL) (Mallinckrodt, St. Louis, Mo.) in a 100 mL round bottom flask
 5 was added dropwise a solution of ω -amino- ω' -carboxy-polyethyleneglycol (0.3 g) and triethylamine (40 mg) in CHCl₃ (20 mL). The resulting mixture was stirred for 5 hours at 10°C. After stirring overnight, the reaction mixture was poured into ice water and neutralized with 10% HCl to a pH of about 3 or less. The lower organic layer was removed using a separatory funnel and washed three times with water. The organic layer
 10 was collected and dried (NaSO₄). Filtration and concentration *in vacuo* yield 0.34 g of DPGS- ω -carboxy-PEG as a white solid.

C. Preparation of 3-succinamoyl-oxy-carbonyl-polyethyleneglycol-imino-succinate-1,2-dipalmitoyl-sn-glycerol (DPGS- ω -carboxy-PEG-succinimide).



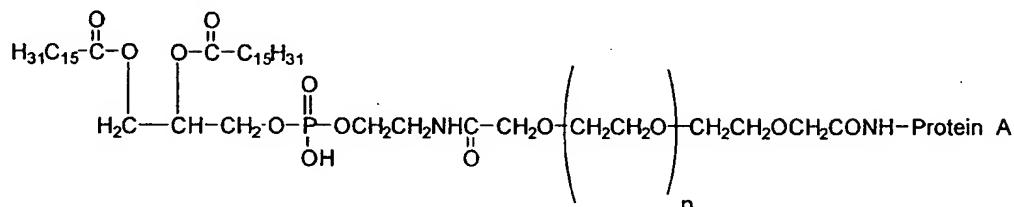
To a cooled (0 to 5°C) solution of DPGS- ω -carboxy-PEG (200 mg) from
 15 Step B, N-hydroxysuccinimide (6 mg), DMAP (2 mg) and acetonitrile (40 mL) in a 250 mL round bottom flask was added dropwise a solution of DCC (12 mg) in acetonitrile (10 mL). The resulting mixture was stirred for 5 hours and the white solid which formed (dicyclohexylurea) was removed by filtration. The filtrate was concentrated *in vacuo* to afford 200 mg of DPGS- ω -carboxy-PEG-succinimide as a white solid.

D. Preparation of DPGS-PEG-Protein A conjugate.

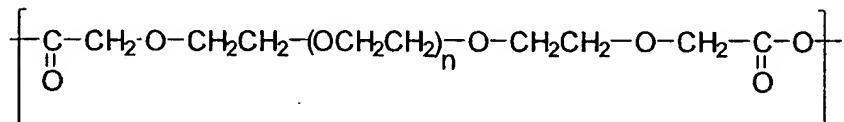
To a cooled (5 to 10°C), stirred solution of Protein A (Sigma Chemical Co., St. Louis, MO) (20 mg) in aqueous buffer (20 mL) at a pH of 8.5 was added dropwise a solution of DPGS- ω -carboxy-PEG-succinimide from Step C (4 mg) and acetonitrile (10 mL). The temperature of the resulting mixture was equilibrated to room temperature and the reaction mixture was stirred for about 48 hours. The mixture was concentrated *in vacuo* and the residual salts were dialyzed away using a dialysis bag having a molecular weight cutoff of about 3500, equilibrated against water. The resulting dialyzed solution was frozen and lyophilized to yield 12 mg of the title material (DPGS-PEG-Protein A conjugate) as a white solid.

Example 14

This example is directed to the preparation of DPPE-PEG-protein A conjugate, which has the following formula.



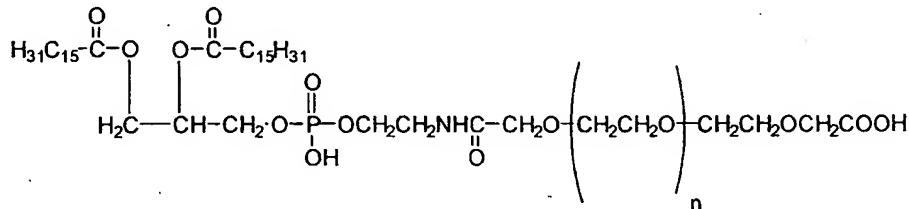
A. Preparation of ω,ω' -dimethylenecarboxy-polyethyleneglycol anhydride.



To a cooled (0 to 5°C) solution of ω,ω' -dimethylenecarboxy-polyethyleneglycol (0.34 g) and CHCl₃ (20 mL) in a 100 mL round bottom flask was added a solution of DCC (0.02 g) in CHCl₃ (5 mL). This solution was stirred overnight, and the resulting white solid precipitate (dicyclohexylurea) was removed by filtration. The filtrate was concentrated *in vacuo* to provide 0.3 g of the anhydride as a white solid.

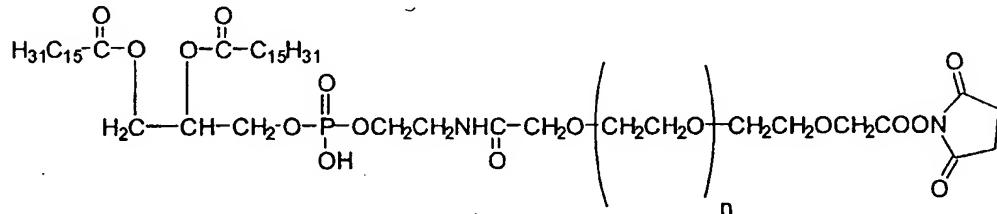
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B. Preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N-carbonyl-methylene- ω '-carboxy-polyethyleneglycol (DPPE- ω -carboxy-PEG).



To a cooled (0 to 10°C) solution of the anhydride from Step A (0.3 g) and CH₂Cl₂ (10 mL) in a 100 mL round bottom flask was added a solution of DPPE (0.07 g) and triethylamine (0.05 g) in CH₂Cl₂ (15 mL). After stirring overnight, the reaction mixture was poured into ice water and acidified with 10% HCl to a pH of about 3 or less. The bottom organic layer was then separated using a 250 mL separatory funnel and dried (NaSO₄). Filtration and concentration of the CH₂Cl₂ solution *in vacuo* yielded 0.45 g of DPPE- ω -carboxy-PEG as an off-white solid.

10 C. Preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethylamine-N-carbonylmethylene-polyethyleneglycol-succinimide (DPPE- ω -carboxy-PEG-succinimide).



To a cooled (0 to 5°C) solution of DCC (3 mg) and acetonitrile (2 mL) was added a solution of DPPE- ω -carboxy-PEG from Step B (60 mg), N-hydroxy-succinimide (1.8 mg) and DMAP (0.2 mg) in acetonitrile (6 mL). After stirring for 3 hours at 0 to 5°C, the temperature of the reaction mixture was equilibrated to room temperature. After stirring overnight, the white solid precipitate which formed (dicyclohexylurea) was removed by filtration, and the filtrate was concentrated *in vacuo* to yield 60 mg of DPPE- ω -carboxy-PEG-succinimide.

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D. Preparation of DPPE-PEG-protein A conjugate.

To a cooled (5 to 10°C) solution of Protein A (20 mg) in 15 ml aqueous buffer (15 mL) at a pH of 8.5 was added dropwise DPPE- ω -carboxy-PEG-succinimide from Step C (4 mg) in acetonitrile (8 mL). The temperature of the reaction mixture was
5 equilibrated to room temperature and stirring was continued for 48 hours. The mixture was concentrated *in vacuo* and the remaining salts were dialyzed against water using a dialysis membrane having a molecular weight cutoff of about 3500. The aqueous dialyzed sample was lyophilized to yield 15 mg of the title product (DPPE-PEG-protein A conjugate) as a white solid.

10

Example 15

This example is directed to the preparation and use of a targeted vesicle composition for targeting epithelial cells.

A. Preparation of vesicle composition.

15 The DPGS-PEG-Protein A conjugate product from Example 13 (1 % by weight) was combined with a dried lipid mixture of DPPC (82 mole %), DPPA (10 mole %), and DPPE (8 mole %). This dry lipid mixture was hydrated and lyophilized on a Labconco (Kansas City, MO) Lyph-Lock 12 lyophilizer. The lyophilized mixture was resuspended in normal saline:propylene glycol:glycerol (8:1:1) at a lipid concentration of 1
20 mg/mL. The mixture was aliquoted into 2 mL Wheaton vials (Millville, NJ). The vials were capped and the headspace in the vials was replaced with perfluorobutane gas (Flura, Newport, TN). The vials were then shaken for one minute on an Espe Capmix® (Seefeld, Germany) to provide gas-filled vesicles. Rabbit anti-keratin antibody (Calbiochem, San Diego, CA) (100 μ L) was added to a sample of the vials and these vials were inverted to
25 mix the antibody with the gas filled vesicles. The vials were incubated at room temperature for about 1 hour. A bicinchoninic acid protein assay (Pierce, Rockford, IL) was performed on the vesicle compositions both before and after washing with PBS. This assay showed that the anti-keratin antibody was bound to the vesicles via Protein A and remained bound during washing.

B. Targeting experiments with the vesicle composition.

HeLa cells (an epithelial cervical cancer cell line which express keratin) were plated in flat sided tissue culture tubes (Nunc, Roskilde, Denmark) in EMEM media (Cellgro, Washington, DC). The cells were grown overnight and aliquots of vesicle compositions were added to each tube. The vesicle compositions employed were: (i) vesicles from Example 6; (ii) vesicles prepared in Step A which contained no rabbit anti-keratin antibody; and (iii) vesicles prepared in Step A which contained rabbit anti-keratin antibody. Only the vesicles in (iii), which included the antibody, binded to the HeLa cells.

Example 16

10 A pegylated lipid will be prepared from distearoylphosphatidyl-ethanolamine (DSPE), (Avanti Polar Lipids, Alabaster, AL) and α -amino, ω -carboxy-PEG3400 (Shearwater Polymer, Huntsville, AL). The amino group will be protected by combining in water:dioxane (1:1, v:v) α -amino ω -amido PEG and one molar equivalent each of t-Boc-anhydride (Bachem, Gardena, CA) and triethylamine (Aldrich Chemical, Milwaukee, WI). The resulting solution will be stirred overnight and the dioxane will be removed *in vacuo*. To the aqueous residue will be added ten volume equivalents, relative to water, of ethyl acetate (Mallincrodt, St. Louis, MO), and this mixture will be cooled in an ice bath. The pH will be adjusted to pH 2 with aqueous sulfuric acid (2 N) and the aqueous layer will be separated using a separatory funnel. The ethyl acetate layer will be washed with brine, dried ($MgSO_4$) and concentrated *in vacuo* to yield a syrup.

20 The yellow oil, DSPE-t-Boc- α -amino, ω -amido-PEG3400, one molar equivalent of diisopropylcarbodiimide (DIC) (Sigma Chemical Co., St. Louis, MO) and dry dimethylformamide (DMF) will be combined. The solution will be stirred and one molar equivalent each of diisopropylethylamine (DIEA), (Sigma Chemical Co., St. Louis, 25 MO) and hydroxylbenzotriazole (HOBT) will be added. The solution will be stirred for two hours and concentrated *in vacuo*. The concentrated residue DSPE-t-BOC- α -amino, ω -amido-PEG3400 will be combined with trifluoroacetic acid and methylene chloride (4:6, v:v) and the resulting mixture will be stirred an additional 20 minutes. The solution will be neutralized with DIEA and concentrated *in vacuo*. This residue will be resuspended in 30 methylene chloride and aqueous hydrochloric acid will be added until a pH of 2 is

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obtained. The aqueous layer will be separated and the pH will be adjusted with 1.0% NaOH to a pH of 10. To this aqueous mixture will be added ethyl acetate which will be separated, washed with saturated brine, and dried ($MgSO_4$). The ethyl acetate solution will be concentrated *in vacuo* to yield a yellow oil.

- 5 To a solution of the yellow oil, DSPE- α -amino, ω -amido-PEG3400 in DMF will be added one equivalent of N-bromosuccinimide (NBS), (Sigma Chemical, St. Louis, MO). After stirring for 3 hours, the reaction mixture will be concentrated overnight *in vacuo*. To a suspension of the resulting concentrated residue in a phosphate-buffered saline (PBS) at a pH of about 7 will be added anti-carcinoembryonic antigen (anti-CEA) 10 which is a monoclonal antibody of mouse/human chimeric origin. After stirring overnight, the solution will be concentrated with an Amicon filter concentrator (Amicon, Beverly, MA) to a volume of ~300 mL. The concentrated solution will be purified by size exclusion chromatography to yield the final product.

Example 17

- 15 A vesicle formulation will be prepared by repeating Example 6, except that about 1 nanomole of recombinant human growth hormone will be added to the lipid composition, prior to vesicle formation.

Example 18

- Example 17 will be repeated except that the DPPA is replaced with the 20 cationic lipid dipalmitoylphosphocholine (Avanti) and the DPPC is replaced with the neutral lipid DPPE. This will provide cationic vesicles to which bioactive agents, especially genetic material, such as the gene for vascular endothelial growth factor (VEGF), can be bound. The incorporation of a targeting ligand, such as an antimyocardial antibody lipid conjugate, will provide targeted vesicles which can be directed to the 25 infarcted and/or ischemic tissue. A burst of high energy ultrasound, for example, 1 MHz continuous wave 200 mW, can be applied with varying pulse duration to promote the rupture of the vesicles. As a result, the VEGF can be substantially delivered directly to the infarcted and/or ischemic tissue.

Example 19

This example is directed to the preparation of a vesicle composition targeted for neuroendocrine tumors of the pancreas.

- Example 16 will be repeated except that Somatostatin peptide will be employed instead of anti-CEA. A therapeutic anti-cancer agent, Streptozocin, will also be incorporated in the vesicle composition. The resulting vesicle composition will be injected intravenously into a patient who has been diagnosed with a neuroendocrine tumor. The vesicles will be preferentially absorbed in the region of the pancreas proximate the tumor, thereby resulting in the enhanced delivery of the anti-cancer agent to the tumor.

10 Example 20

Example 15 was repeated except that, in addition to HeLa cells, the targeted vesicles were also added to an epithelial cell line comprising mouse normal liver cells. Only the vesicles containing the antibodies were bound to the liver cells.

Example 21

- 15 This example is directed to the preparation and use of a vesicle composition targeted for myocardial cells (cardiomyocytes).

A. Preparation of targeted vesicles.

- Example 15 was repeated except that rabbit anti-keratin antibody was replaced with rabbit anti-human skeletal myosin antibody (Biomakor™, Kiryat Weizmann, 20 Rehovot, Israel) (100 µL).

B. Targeting of myocardial cells.

- Rat heart myoblasts (American Type Culture Collection, Rockville, MD) were plated in flat sided tissue culture tubes (Nunc, Roskilde, Denmark) in EMEM media (Cellgro, Washington, DC). The cells were grown overnight and aliquots of vesicle 25 compositions were added to each tube. The vesicle compositions employed were: (i) vesicles from Example 6; (ii) vesicles prepared in Step A above which contained no rabbit anti-myosin antibody; and (iii) vesicles prepared in Step A above which contained rabbit

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anti-myosin antibody. Only the vesicles in (iii), which included the antibody, binded to the myoblast cells.

Example 22

This example is directed to the preparation and use of a vesicle composition
5 targeted for myocardial cells (cardiomyocytes).

A. Preparation of targeted vesicles.

Example 15 was repeated except that rabbit anti-keratin antibody was replaced with rabbit anti-myosin (skeletal) antibody (Accurate Chemical, Westbury, NY) (100 µL).

10 B. Targeting of myocardial cells.

H9C2, a rat cardiac myocite line which expresses cardiac myosin, was employed. (Skeletal muscle myosin and cardiac muscle myosin are substantially similar.) The control cell line was INT407, which is a human intestinal cell line. The cells were plated in flat sided tissue culture tubes (Nunc, Roskilde, Denmark) in EMEM media
15 (Cellgro, Washington, DC). The cells were grown overnight and aliquots of vesicle compositions were added to each tube. The vesicle compositions employed were: (i) vesicles from Example 6; (ii) vesicles prepared in Step A above which contained no rabbit anti-myosin antibody; and (iii) vesicles prepared in Step A above which contained rabbit anti-myosin antibody. Three experimental cell types were employed: (a) INT407; (b)
20 H9C2; and (c) H9C2 that was exposed to 10% glucose for 10 minutes to shock the cells and open pores to allow binding of the antimyosin to the cytoplasmic myosin. No binding was observed with vesicle compositions (i) or (ii) in any of cell types (a), (b) or (c). Vesicle composition (iii) also did not bind to cell type (a). Some binding of vesicle composition (iii) to cell type (b) was observed, whereas substantial binding of vesicle
25 composition (iii) to cell type (c) was observed.

Example 23

Dipyridamole (*see, e.g., The Merck Index, 10th Ed., p. 489 (1983)*) is a bioactive agent in the context of the present invention in that it is a coronary vasodilator. Dipyridamole is also a targeting ligand within the context of the present invention since it
5 can bind to one or more receptors in heart tissue, and can also be attached to phospholipids to provide membrane compatible derivatives. Specifically, N-succinyl-DPPE will bind with dipyridamole using dicyclohexylcarbodiimide and a catalyst as a condensation agent to form N-dipyridamolylsuccinate-DPPE. This lipid bound dipyridamole derivative will be employed in a lipid composition for the preparation of vesicles for targeting heart
10 tissue.

Example 24

A balloon catheter will be placed percutaneously via a femoral arterial access into the left circumflex coronary artery of a laboratory animal. The balloon will be inflated for a period of three hours, preventing blood flow to the anterolateral myocardium
15 and resulting in a myocardial infarction. Echocardiography will be performed with a 5 MHz transducer and will reveal a persistent wall motion abnormality, as well as myocardial thinning of the anterolateral wall of the left ventricle. The balloon will be deflated and a dose of 30 μ L/Kg of anti-cardiomyosin antibody labeled vesicles similar to those prepared, for example, in Step A of Example 22, filled with dodecafluoropentane gas
20 will be injected intravenously. Echocardiography will be performed thirty minutes after injection. Imaging will show an area of increased echogenicity in the anterolateral wall corresponding to the region of the infarct. The periphery of the infarct (reperfused region) appears brightest with less intensity centrally (region of persistent absence of flow). In comparison, the central ventricular cavity is relatively dark, with a majority of the vesicles
25 having been cleared by this time.

Example 25

A patient suffering from chest pains will be admitted to the emergency room of a hospital. An ultrasound study will be performed and will indicate a wall motion abnormality in the anterior wall of the left ventricle. The patient will be administered a

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dose of 5 μ L/Kg of anticardiomysin antibody labeled vesicles similar to those prepared, for example, in Step A of Example 22, filled with perfluorobutane gas. Ten minutes after administration, ultrasound imaging will be repeated and will show an area of increased echogenicity in the left ventricle anterior wall. This will confirm the diagnosis of
5 myocardial infarction, and the patient will be treated with tissue plasminogen activator.

Example 26

A patient will undergo a stress echocardiography examination to diagnose the presence or absence of coronary artery disease. During peak exercise, the patient will be administered 5 μ L/Kg of perfluoropropane filled vesicles labeled with dipyridamole as
10 described in Example 23 above. The dipyridamole will be combined with the vesicles through the use of (a) a bifunctional linking group; (b) a small spanning molecule; (c) a Schiff's base with or without reductive amination; or (d) a maleimidyl linker. The vesicles will bind to perfused regions of myocardium. As the background vesicles clear, which takes about 5 minutes, the labeled vesicles will persist within the myocardium. Delayed
15 imaging will show the ischemic myocardium affected by coronary artery disease (CAD) as a hypointense region surrounded by bright, normally perfused myocardium. The absence of any appreciable background vesicles within the cardiac chambers eliminates the problem with shadowing and facilitates making the diagnosis of CAD.

Example 27

20 This example demonstrates the targeted expression of a protein using a vesicle composition and methods within the scope of the present invention.

Three Sprague-Dawley rats, identified herein as rats (A), (B) and (C), were anesthetized with Ketamine acepromazine. pSV β -gal, a plasmid containing the β -galactosidase gene with the SV40 promoter and enhancer genes, was combined with the
25 cationic lipid compound prepared in Example 2. The plasmid and cationic lipid compound mixture was incubated for 15 minutes at room temperature. cGMP and the resulting incubated material was then added to a blend of DPPC, DPPA and DPPE-PEG5000 at a respective mole % ratio of 82:10:8 in normal saline:glycerol:propylene glycol (8:1:1). Vesicles were prepared by shaking the resulting mixture on a WIG-L-BUGTM for one

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minute at 3200 rpm. The resulting vesicle composition was injected into rats (A), (B) and (C). The dosages were as follows.

<u>Rat</u>	<u>Dose pSVβgal (μg)</u>	<u>Dose Cationic lipid (μg)</u>
(A)	5	30
5 (B)	5	30
(C)	25	150

During injection of the vesicle composition, Rats (B) and (C) were exposed to ultrasound energy from a therapeutic ultrasound machine (1.0 Mhz, Rich-Mar model 25, Rich-Mar Corporation, Inola, OK). The ultrasound was directed towards the inside of the left hind leg during the injection and for one minute post flush. Rat (A) did not receive any ultrasound treatment. After 48 hours, the rats were euthanized by asphyxiation with CO₂. The left and right leg muscle and skin were removed from each rat. The hearts, livers and kidneys were also removed. The excised tissues were fixed for 72 hours in 2% formalin. After fixing, the presence of β -galactosidase activity was assayed by soaking the tissue in a solution containing X-gal, potassium ferrocyanide and potassium ferricyanide. The tissues were stained, inspected and photographed. Expression was seen broadly dispersed throughout the leg tissues, particularly in the endothelial cells, muscle and skin, in rat (A). Inspection of the excised tissues from rat (B) showed expression in similar cell types but only at the site of ultrasound application. Rat (C) showed a point expression in the target leg and dispersed expression in the leg not targeted.

Example 28

This example is directed to the preparation of various lipid compositions. DPPC, DPPA and DPPE-PEG5000 were blended at a respective mole ratio of 82:10:8. This lipid mixture is referred to herein as Composition (A). A portion of 25 Composition (A) was mixed with the cationic lipid compound prepared in Example 2 at a 10:1 (w/w) ratio in distilled deionized water. This latter mixture is referred to herein as Composition (B). Compositions (A) and (B) were introduced into 1 ml serum vials. The vials were evacuated with a Sargent Welch vacuum pump (Skokie, IL) and the headspaces

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were replaced with perfluoropropane gas (Flura Chemical Corp, Newport, TN). The vials were agitated with a Crescent Dental WIG-L-BUG™ 311OB (Lyons, IL) mechanical shaker. After shaking, heparin (Elkin Sinns Inc., Cherry Hill, NJ) was added to a portion of the vials at an approximate molar ratio of lipid to heparin of 5:1. The mixture of
5 heparin and Composition (A) are referred to herein as Composition (C). The mixture of heparin and Composition (B) are referred to herein as Composition (D).

Recombinant human basic fibroblast growth factor (BFGF) (Sigma, St. Louis, MO) was then added to Compositions (A), (B), (C) and (D), which are respectively referred to herein as Compositions (E), (F), (G) and (H). The BFGF was added to the
10 compositions which contained heparin (Compositions (C) and (D)) at a molar ratio of 5:1 (BFGF:heparin). At least three samples from each of Composition (A) to (H) were sized using an Accusizer 770 (Particle Sizing Systems, Santa Barbara, CA). Sizing showed no difference in the sizes of vesicles in Compositions (A) to (G). However, the vesicles in Composition (H), which contained cationic lipid, heparin and BFGF, had a smaller mean
15 size.

Compositions (E) to (H) were then analyzed using a native polyacrylamide gel (protein gel PAGE mix, Boehringer Mannheim, Indianapolis, IN) to identify binding properties of heparin and BFGF. The gel was run on a Hoefer SE400 slab gel apparatus (Hoefer Scientific, San Francisco, CA). The gel was run at a constant current using an
20 electrophoresis power supply (MBP300, IBI, Rochester, NY). The gel was then stained using a rapid Coomassie blue (Eastman Kodak, Rochester, NY) staining method and analyzed visually. Gel electrophoresis confirmed that in Composition (H), the presence of the cationic lipid enhances binding of heparin as well as the BFGF. It is contemplated that the heparin, which is anionic, binds to the cationic lipid, and that the BFGF, which is
25 cationic, binds to the heparin. BFGF in compositions which contain no heparin and cationic lipid migrates during electrophoresis.

Example 29

Example 16 will be repeated, except that vascular endothelial growth factor (VEGF) will be employed instead of anti-CEA antigen.

Example 30

DPPC, DPPA and DPPE-PEG5000 will be combined in a mole percent ratio of 82:10:8 in saline:propylene glycol:glycerol (8:1:1, w/w/w). The total concentration of lipids in the solution will be 1 mg/mL. Recombinant human growth 5 hormone will be added to this mixture at a concentration of 10% by weight, relative to the total weight of lipids. An aliquot (1.5 mL) of this mixture will be placed in a 3 mL volume glass vial and the headspace of the vial will be exchanged with perfluoropropane gas. The vial will be sealed and shaken, resulting in gas filled vesicles which bind human growth 10 hormone and which will be useful for targeting endothelial cells. The mean diameter of the vesicles will be about 3 μ m.

Example 31

Distearoylphosphatidylglycerol (DSPG) and DPPE-PEG 5000 will be combined in sterile water at a molar ratio of 92:8. The concentration of lipid will be about 1 mg/mL. Chitosan will be derivatized with 10 mole percent by weight of basic fibroblast 15 growth factor (bFGF) using amide bonds to attach the amine groups of the chitosan to carboxyl moieties of the bFGF. The derivatized chitosan will be added to the lipid suspension in an amount to provide a substantially equivalent concentration of cationic groups in the chitosan and anionic groups in the DSPG. An aliquot of this mixture (1.5 mL) will be placed into a 3 mL vial and the headspace will be replaced with 20 perfluorobutane. The vial will be sealed and shaken on an ESPE Capmix (Seefeld, Germany) to provide gas-filled vesicles useful for targeting endothelial cells.

Example 32

A pegylated lipid will be prepared from DSPE and α , ω -bis(carboxymethyl)-PEG2000 (Shearwater Polymers, Huntsville, AL) to provide MeO₂C-PEG-DSPE. This lipid will be purified by column chromatography (silica gel) to provide 25 the free acid (HO₂C-PEG-DSPE), after which VEGF will be linked to the free carboxyl group. DPPC, DSPA, DSPE-PEG and DSPE-PEG-VEGF will be combined, at a molar ratio of 82:10:6:2, in saline:glycerol:propylene glycol (8:1:1, w/w/w), at a total lipid concentration of 1.5 mg/ml. An aliquot of this mixture (1.5 mL) will be placed into a

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sterile 3 mL vial and the headspace of the vial will be replaced with perfluoropentane gas at 30°C and the vial will be sealed. The vial will be shaken at 30°C for about 90 seconds at 3,200 rpm on an ESPE Capmix (Seefeld, Germany) resulting in perfluoropentane gas filled targeted vesicles.

5 **Example 33**

DSPC, cholesterol and DPPA will be combined, at a mole percent ratio of 65:27:8, in a solution of saline:propylene glycol:glycerol (8:1:1 w/w/w). The total lipid concentration will be 5 mg/mL. An aliquot of this mixture (1.5 mL) will be placed in a sterile vial and the headspace will be replaced with perfluorobutane gas. The vial will be
10 sealed and shaken for about 5 minutes on an ESPE Capmix (Seefeld, Germany) to provide a vesicle composition within the scope of the present invention. A Watanabi rabbit which had been fed a high cholesterol diet, will be administered i.v. a dose of 0.10 mL/Kg of the vesicle composition. Ultrasound imaging will be performed about 25 minutes after administration and will show that the vesicles accumulated in regions of endothelial cells
15 affected by atherosclerotic plaque. This will illustrate that the vesicle compositions of the present invention are useful in detecting atherosclerotic regions of endothelial cells.

Example 34

Example 33 will be repeated except that among the lipids used will be cholesterolamine linked covalently via an amide linkage to DPPE-PEG-carboxylate. The
20 lipid mixture will then comprise DPPC, DPPE-PEG5000, DPPE-PEG-cholesterolamine and DPPA which will be combined at a mole percent ratio of 82:7:1:10.

Example 35

Example 33 will be repeated except that the lipid concentration will be increased to about 5 mg/mL and the suspending medium will comprise a solution of
25 normal saline, trehalose (10 mg/mL) and pluronic F-68 (10 mg/mL). This suspension of lipids will be microemulsified using a Microfluidizer (Microfluidics, Newton, MA) at 16,000 psi for a total of ten passes. The resulting vesicle composition will be lyophilized and the headspace of the lyophilization chamber will be restored gradually to ambient

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pressure over a period of 72 hours by slowly instilling perfluorobutane gas. The resulting gas-filled lyophilized vesicles will be stored as a dry powder until use.

Example 36

- Example 35 will be repeated except that the lipid concentration will be increased to about 25 mg/mL, and the suspending medium will comprise a solution of normal saline, sorbitol (20 mg/mL) and pluronic F-68 (20 mg/mL). The lipid suspension will be cooled to 4°C and to this mixture will be added perfluoropentane to provide a perfluoropentane concentration of 8 µL per mL of solution. This mixture will be passed through a microfluidizer maintaining the temperature at 4°C for 20 passes at 16,000 psi.
- This will provide a vesicle composition of perfluoropentane-filled vesicles. This composition will be injected i.v. into a patient and will form gas filled vesicles *in vivo* useful for targeting endothelial cells. Gas-filled vesicles can also be obtained prior to i.v. injection by warming the vesicle composition to above about 30°C and shaking, for example, on an ESPE Capmix (Seefeld, Germany) or other shaker or amalgamator device,
- or by withdrawing the plunger of a syringe which is filled with the composition of perfluoropentane-filled vesicles so as to decrease the pressure and convert the perfluoropentane liquid into perfluoropentane gas.

Example 37

- 12-Hydroxydodecanoic acid will be esterified with methacryloyl chloride and the ester will be converted to the anhydride to yield 12-(methacryloyloxy)dodecanoic anhydride. L-alpha-Glycerophosphocholine derived from egg lecithin will be acylated with the 12-(methacryloyloxy)dodecanoic anhydride to yield bis[12-(methacryloyl)oxydodecanoyl]-L-alpha-phosphatidylcholine (1). Enzymatic hydrolysis of (1) with phospholipase A₂, derived from crude rattle snake venom (*Crotalus adamanteus*), will be followed by acylation with palmitoyl anhydride to yield 1-[2-(methacryloyloxy)dodecanoyl]-2-palmitoyl-L-alpha-phosphatidylcholine (2).

The above (2) will be combined with normal saline at a lipid concentration of 3 mg/mL. To this mixture will be added DSPE-PEG-VEGF at a concentration of 0.30 mg/ml. An aliquot of the mixture (1.5 mL) will be placed in a sterile 3 mL vial. The

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headspace of the vial will be evacuated and replaced with perfluoropropane gas and the vial will be sealed and wrapped with light impermeable foil. The vial will be shaken for about 3 minutes on an ESPE Capmix (Seefeld, Germany) to provide gas-filled vesicles. The foil will be removed and the vesicle composition will be irradiated with light (254 nm)

5 to provide polymerized gas-filled vesicles bearing VEGF for targeting endothelial cells.

Example 38

Example 37 will be repeated, except that lipid (2) will be combined with DSPE-PEG (see Example 8). VEGF will be admixed with this lipid mixture prior to shaking on the ESPE Capmix (Seefeld, Germany) rather than being covalently attached to

10 the DSPE-PEG. After photopolymerization, the VEGF will be embedded directly into the polymeric lipid membranes which coat the vesicles.

Example 39

This example is directed broadly to the preparation of albumin vesicles which are internally crosslinked and which comprise surfaces that are modified by the

15 attachment of polyoxy(C₁₋₄) alkylene chains as described in Published International Application WO 95/00126, the disclosures of which are hereby incorporated by reference, in their entirety. The termini of the alkylene chain modifiers will contain a reactive group, unlike the termini of the alkylene chains in WO 95/00126, which are ether groups. The reactive groups will be included for binding an endothelial targeting ligand.

20

Example 39A

An aqueous solution of 5% human bovine serum albumin (5 mL) will be introduced into an ultrasonic reaction vessel at a speed of 500 mL/min. The mixture will be sonicated using a Sonifier B-30 (Branson, FRG) for about 15 minutes at an energy flux of 100 Watt/cm². The temperature will be maintained at 22°C by refrigeration of the

25 reaction vessel. If desired, a bioactive agent, such as a drug, may be included in this mixture. Crosslinking will be initiated by adding to the albumin solution a solution of CH₂Cl₂ (0.2 mL) saturated with glutaraldehyde. The resulting solution will be stirred for about 1 hour. After washing with methanol, followed by acetone and finally with n-

hexane, the crosslinked albumin vesicles will be obtained as a brown powder which will be dried under vacuum for about 24 hours. The size of the resulting vesicles will be determined by a Nicomps laser light particle scattering system at approximately 200 nm.

Example 39B

5 Albumin vesicles having a larger diameter than the vesicles prepared in Example 39A will be prepared by replacing the high intensity emulsifying process with a 4 bladed impeller and using higher concentrations of albumin, for example, from about 10 to about 25%. Crosslinking will be carried out as described above in Example 39A.

Example 39C

10 This example is directed to the preparation of gas-filled albumin vesicles. Albumin vesicles will be prepared by placing 5 mL of 5% albumin in a sterile vial and filling the headspace of the vial with perfluorobutane gas. The vials will be shaken for about 5 minutes at 3,300 rpm on an ESPE Capmix (Seefeld, Germany). Crosslinking will be carried out as described above in Example 39A.

Example 39D

This example is directed to the preparation of gas-filled albumin vesicles. Albumin vesicles will be prepared by sonicating a vial containing a 5% solution of human serum albumin and a headspace of perfluoropropane gas. This sonication will involve immersing minimally the sonicator horn tip into the liquid interface 20 for about 5 minutes at medium power setting (Heat Systems Probe, Farmingdale, NY). The albumin vesicles will be crosslinked as described above in Example 39A.

Example 39E

This example is directed to coupling heterobifunctional PEG to the albumin vesicles prepared above.

25 The ω carboxy group of α -amino, ω -carboxy-PEG5000 (Shearwater Polymers) will be protected with a suitable ester functionality, for example, a benzyl ester. The amino group of the PEG will be bonded covalently to the albumin vesicles to provide

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- a 10:1 weight ratio of albumin to PEG by activating the appropriate amino acid moieties on the protein, such as, for example, the aspartic acid or glutamic acid moiety, with 1,1-carbonyldiimidazole. After covalent bonding of the PEG to the albumin via an amide linkage, the benzyl ester group will be removed. aFGF will be attached to the free
- 5 carboxyl group by activating the carboxylate end of the PEG with 1,1-carbonyldiimidazole. This provides the endothelial targeting ligand bound to the albumin vesicles via the PEG linkers.

- Alternatively, coupling may be accomplished by activating the appropriate amino acids of aFGF, for example, aspartic acid or glutamic acid, with
- 10 carbonyldiimidazole, followed by the addition of carboxyl group protected α -amino, ω -carboxy-PEG. The protected carboxyl group will be deprotected, and the unprotected carboxyl group will be activated with carbonyldiimidazole. The activated aFGF-PEG will be combined and reacted with the albumin vesicles.

- Gas will be instilled into the vesicles by careful dehydration of the vesicles
- 15 under vacuum and gradual restoration to ambient pressure using a gas, such as perfluorobutane. One or more surfactants, for example, pluronic F-68, may be added prior to the dehydration and gas instillation step.

Example 40

- This example is directed to the preparation of gas filled clathrates.
- 20 The ω carboxy group in α -hydroxy, ω -carboxy-PEG2000 will be protected with a suitable ester functionality, for example, a benzyl ester. The protected compound will be reacted with one equivalent of POCl_3 in Et_3N and CHCl_3 at 0-5 °C to convert the hydroxy group to the phosphorous chloride ester. This compound will then be reacted with excess NaHCO_3 to produce the corresponding sodium phosphate ($\text{PEG-PO}_4\text{Na}_2$).
- 25 This salt will be acidified with HCl , pH 3.0, to provide the corresponding acid ($\text{PEG-PO}_4\text{H}_2$). This product will be dialyzed against purified water using a membrane having a molecular weight cutoff of about 100.

- After dialyzing, the above phosphoric acid product (2 g) will be combined with disodium dihydrogen pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$) (8 g) in water (100 mL). This
- 30 solution will be introduced into a Buchi spray drying apparatus and spray dried. The

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resulting particles will be baked at 120°C under vacuum for about 1 hour to provide hollow PEG-coated pyrophosphate vesicles. These vesicles will be resuspended in an aqueous medium and the benzyl protecting group will be removed. The deprotected carboxyl group of the PEG will be activated with 1,1-carbonyldiimidazole and the

5 resulting compound will be coupled to a monoclonal antibody for ELAM for targeting endothelial cells. To an aqueous medium containing the targeted vesicles will be added DDPC and pluronic F-68 to provide a concentration of 1 mg/mL and 5 mg/mL, respectively. The aqueous medium containing the vesicles will be heated to about 45°C, agitated gently by hand and dried under vacuum using a rotary evaporator while

10 maintaining this temperature. The resulting dried material will be introduced into a suitable container and lyophilized. Perfluorobutane gas will be instilled gradually in the headspace of the container over a period of about 48 hours until ambient pressure is achieved. The resulting gas filled pyrophosphate clathrates will be stored as a lyophilized powder until use.

15 **Example 41**

A 0.25 M Ca^{+2} solution will be prepared by dissolving $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (3.68 g) in distilled water (100 mL). The pH of this solution will be adjusted to pH 11 using 0.1 M NaOH. A 0.74 M solution of potassium dihydrogenphosphate (KH_2PO_4) will be prepared by dissolving 0.75 g of the salt in distilled water (10 mL). To this solution will be added

20 1.75 g of bifunctional PEG, α -phenylcarboxy ester, ω -phosphoryl-PEG5,000. The pH will be adjusted again with 0.1 M NaOH to pH 11. The potassium dihydrogenphosphate/PEG phosphate solution will be added dropwise to a vigorously stirred solution of CaCl_2 . The resulting precipitate will be sized by single particle optical sizing and by optical microscopy. The diameter of the particles will be in the range of about 1 to 2 μm . The

25 particles will be filtered and resuspended in normal saline and the phenyl group will be removed from the carboxy group. The carboxy group will be activated with 1,1-carbonyldiimidazole and this will be reacted with a monoclonal antibody specific for ELAM. The monoclonal antibody may be prepared by conventional techniques according to the methods of Kohler and Milstein, *Nature* 1975, 256:495, the disclosures of which

30 are incorporated herein by reference, in their entirety. The particles will be suspended in a

solution of pluronic F-68 (20 mg/mL) and this suspension will be introduced into a suitable container and lyophilized. The headspace of the container will be equilibrated gradually to ambient pressure with perfluoropropane gas to provide the hydroxyapatite gas clathrates.

5 **Example 42**

In vivo experiments in rats were conducted which demonstrate the high effectiveness of the cationic lipid compounds of Example 3 to deliver genetic material intracellularly. The experiments demonstrate also the effectiveness of using ultrasound energy for targeting specific tissue *in vivo* with vesicle compositions containing genetic material. Plasmid pSV β-gal (Promega, Madison, WI), which contains the β-galactosidase gene, and the cationic lipid compound prepared in Example 3 were combined by mixing. The resulting mixture was injected into each of three Sprague Dawley rats (rats (A), (B) and (C)) via the tail vein. Rat (A) was not subjected to ultrasound. Ultrasonic energy was applied to the inside of the hind leg during injection for each of rats (B) and (C). After 48 hours, the rats were euthanized and the tissues were removed. The tissues were fixed for 72 hours in 2% formalin, sliced thin and placed in an X-gal solution. After 16 hours at 37°C, the tissues were inspected. The tissue from rat (A) exhibited a blue color which is indicative of general transfection. The tissue from rats (B) and (C) exhibited blue color only at the site where ultrasound energy was applied. This indicates that localization of gene expression can be achieved with the compounds and methods of the present invention.

Example 43

Fresh human blood was applied to gauze strips (Tillotson Health Care, Bedford, NH) and allowed to clot. Tygon tubing was cast into a 1% agarose (Boehringer Mannheim Biochemical, Indianapolis, Indiana) standoff pad. The strips bearing the blood clots were inserted into the Tygon tubing. Inline mixing elements (Cole-Parmer, Chicago, IL) were also inserted into the tubing to create a turbulent flow. Phosphate buffered saline (Boehringer Mannheim Biochemical) was pumped across the gauze strip using a MasterFlex peristaltic pump (Cole-Parmer). Ultrasound imaging was carried out using an

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Acoustic Imaging 5200 ultrasound machine with a 7.5 MHz peripheral vascular transducer (Phoenix, AZ). The transducer was fixed to a ring stand and imaging was carried out from underneath the standoff pad. After the unbound clot was washed from the strip, 1 mL each of a targeted vesicle composition which targets GPIIbIIIa and the vesicle composition

5 from Example 6 (non-targeted) was injected into the PBS stream at a flow rate of approximately 2 mL/min. The vesicles were allowed to bind at this speed for 2 minutes and then the flow rate was turned up to 6 mL/min to remove unbound vesicles. After washing at 6 mL/min, the targeted vesicles were bound to the gauze strip, while the non-targeted vesicles were washed away. Ultrasound imaging revealed increased echogenicity

10 in the clot with vesicles targeted to GPIIbIIIa.

Videodensitometric analysis was performed offline using a Macintosh 660 AV computer (Apple Computing, Cupertino, CA). The entire area of the clot was selected and the videodensitometry units were measured. The images were captured and analyzed using Image 1.55 (National Institutes of Health, Washington, DC). This analysis involved

15 collecting and superimposing the pre-contrast baseline image with the post-contrast image, and subtracting the pre-contrast image from the post-contrast image. This collection, superimposition, and subtraction were also conducted before and after washing to remove unbound vesicles. The videodensitrometric analysis was conducted using both continuous and intermittent ultrasound. The data obtained in this analysis is set forth in the following

20 table, wherein higher numbers represent improved contrast.

TABLE 4
Videodensitometric Analysis of Targeted Vesicles-
Ultrasound Backscatter Quantitation

CONTINUOUS IMAGING				
	<i>Targeted Vesicles (before wash)</i>	<i>Non-Targeted Vesicles (before wash)</i>	<i>Targeted Vesicles (after wash)</i>	<i>Non-Targeted Vesicles (after wash)</i>
	28.46	17.64	46.01	16.5
	18.44	13.36	39.48	14.44
	14.44	7.62	17.22	7.58
	40.94	16.97	32.85	12.95
	22.86	6.81	19.52	8.08

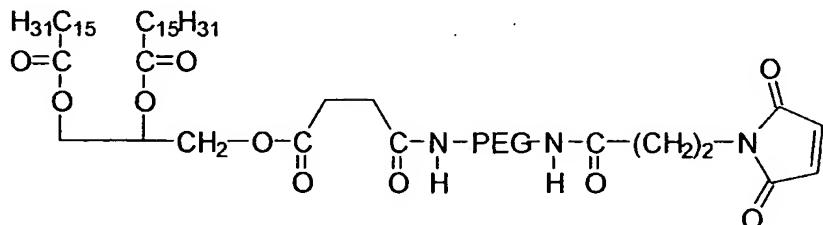
TABLE 4
Videodensitometric Analysis of Targeted Vesicles-
Ultrasound Backscatter Quantitation

Mean	25.57	13.9	33.89	12.87
StDev	11.82	4.59	12.34	3.81
INTERMITTENT IMAGING				
	<i>Targeted Vesicles (before wash)</i>	<i>Non-Targeted Vesicles (before wash)</i>	<i>Targeted Vesicles (after wash)</i>	<i>Non-Targeted Vesicles (after wash)</i>
	16.6	12.06	17.83	12.14
	15.47	16.55	20.35	13.7
	19.32	16.13	21.79	16.35
	20.12	--	22.41	8.68
	17.29	13.7	24.15	13.41
Mean	17.88	14.91	20.6	12.72
StDev	2.2	2.48	2.04	3.2

Inspection of the above table reveals increased backscatter from the targeted vesicles as compared to the non-targeted vesicles. T-tests were conducted which demonstrated a significant increase in videodensity signals for the targeted vesicles as compared to the non-targeted vesicles in both continuous and intermittent imaging $p < 0.01$.

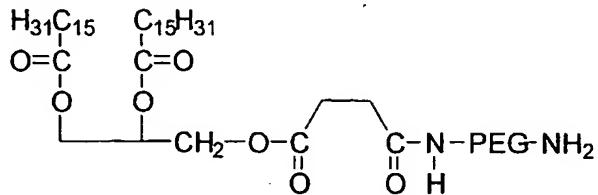
10 **Example 44**

This example is directed to the preparation of DPGS-NH-PEG-NH-maleimide, which has the following formula.



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A. Preparation of dipalmitoyl glycerol succinate-poly(chleneglycol-amine) (DPGS-NH-PEG-NH₂).



Into a round bottom flask (100 mL) was added a solution of DPGS (98 mg, 0.147 mmole, 1 eq.) in CH₂Cl₂. Into a separate round bottom flask (100 mL) was added a
 5 solution of H₂N-PEG-NH₂ (PEG-diamine) (50 mg, 0.147 mmole, 1 eq.) in CH₂Cl₂. To the PEG-diamine solution was added immediately a solution of DCC (31 mg, 0.15 mmole, 1.02 eq.) in CHCl₃ (5 mL). The resulting mixture of DCC and PEG-diamine was added dropwise to the DPGS at about 0 to about 5°C. The resulting reaction mixture was equilibrated with stirring to room temperature. After stirring for 24 hours, distilled-
 10 deionized water (50 mL) was added to the reaction mixture and the resulting white precipitate (dicyclohexylurea) was removed by filtration. The lower organic layer was isolated using a 250 mL separatory funnel and dried (Na₂SO₄). The dried organic solution was filtered and CH₃CN (80 mL) was added to the filtrate. The remaining white precipitate was removed by filtration and the filtrate was concentrated *in vacuo* to yield
 15 0.44 g of DPGS-NH-PEG-NH₂ as a solid product.

IR: 1700 cm⁻¹.

TLC: R_f 0.65.

B. Preparation of DPGS-PEG-NH-PEG-NH-maleimide.

Into a round bottom flask (100 mL) was added a solution of DPGS-NH-
 20 PEG-NH₂ from Step A (0.44 g, 0.13 mmole, 1 eq.) in CH₂Cl₂ (10 mL). To this solution was added a solution of triethylamine (13 mg, 0.13 mmole, 1 eq.) in CH₂Cl₂. To this mixture was added dropwise a solution of 34.6 mg N-maleoyl-β-alanine N-hydroxysuccinimide ester (34.6 mg) in CH₂Cl₂ (10 mL). After stirring overnight at room temperature, deionized-distilled water (30 mL) was added to the reaction mixture and
 25 stirring was continued an additional 3 hours. The lower organic layer was isolated using a

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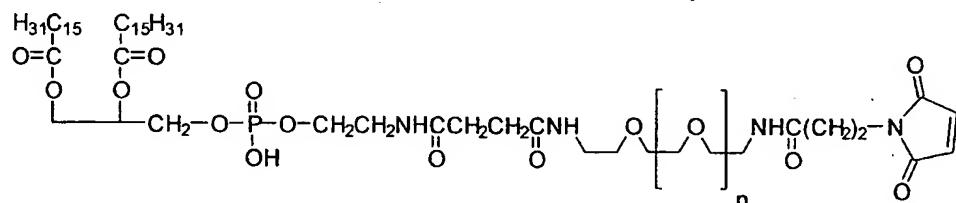
separatory funnel and dried (NaSO_4). The organic layer was filtered and the filtrate was concentrated *in vacuo* to afford 360 mg of the title compound as a wax-like white solid.

IR: 1740 cm^{-1} , 1670 cm^{-1} , 1100 cm^{-1} .

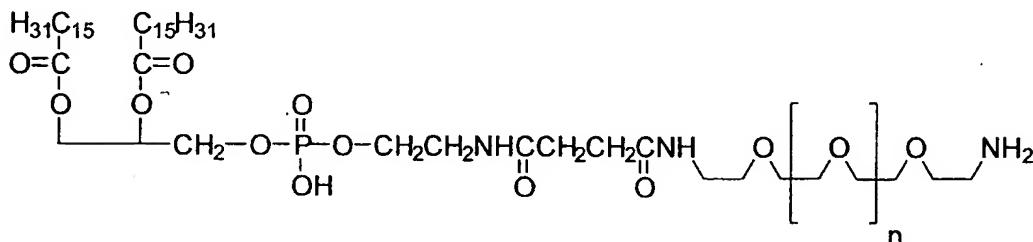
TLC: $R_f 0.75$.

5 Example 45

This example is directed to the preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N-carbonylethylenecarbonyl-imino-polyethylene-glycolethylaminocarbonylethylenemaleimide (DPPE-NH-PEG-NH-maleimide), which has the following formula.



- 10 A. Preparation of 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine-N-carbonylethylenecarbonyliminopolyethyleneglycolethylamine (DPPE-NH-PEG-NH₂).



- To a cooled (0 to 5°C) solution of DPPE succinate (79 mg) and ω,ω' -diaminopolyethyleneglycol (340 mg) in CH_2Cl_2 (20 mL) was added a solution of DCC (22 mg) in CH_2Cl_2 (10 mL). The reaction mixture was stirred for 4 hours at 0 to 5°C and then 15 stirred overnight at room temperature. Water (10 mL) was added, and the reaction mixture was stirred for an additional hour. The lower organic layer was isolated and concentrated *in vacuo* using a rotary evaporator. The residue was redissolved in CH_3CN and the precipitate was removed by filtration. Concentration of the organic solution *in vacuo* yielded 390 mg of DPPE-NH-PEG-NH-maleimide as a white solid.

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B. Preparation of DPPE-NH-PEG-NH-maleimide.

To a cooled (0 to 5°C) solution of DPPE-NH-PEG-NH₂ from Step A (210 mg) and triethylamine (20 mg) in CH₂Cl₂ (20 mL) was added a solution of maleimidopropionic acid N-hydroxysuccinimide ester (26 mg) in CH₂Cl₂ (10 mL). The 5 resulting mixture was stirred for 1 hour at 0 to 5°C, and then stirred overnight at room temperature. Water (10 mL) was added and the resulting mixture was acidified with dilute HCl, with stirring, to a pH of 3. The organic layer was isolated, washed with water (10 mL) and dried (NaSO₄). Concentration to dryness *in vacuo* using a rotary evaporator provided 210 mg of the title compound (DPPE-NH-PEG-NH-maleimide) as a white solid.

10 **Example 46**

Fab' or F(ab')₂ fragments were bound to gas-filled vesicles via a lipid-PEG-maleimide lipid. The Fab' or F(ab')₂ fragments were prepared from anti-skeletal myosin (Sigma Chemical, St. Louis, MO) using Fab' and/or F(ab')₂ kits from Pierce (Rockford, IL).

15 Fab' fragments were prepared by digesting the antibody with papain. This cleaves both Fab' fragments from the Fc region of the antibody. The Fc fragments were then bound to a Protein A column and the Fab' fragments were collected. The Fab' fragments were then lyophilized (Labconco Lyph-Lock 12, Kansas City, MO) and prepared for binding to the lipid-PEG-maleimide lipid.

20 F(ab')₂ fragments were prepared in a similar manner as for the Fab' fragments. However, the enzyme pepsin was used instead of papain. Pepsin cleaves farther in the Fc region, and the two Fab' fragments remain connected. The F(ab')₂ fragments were then lyophilized.

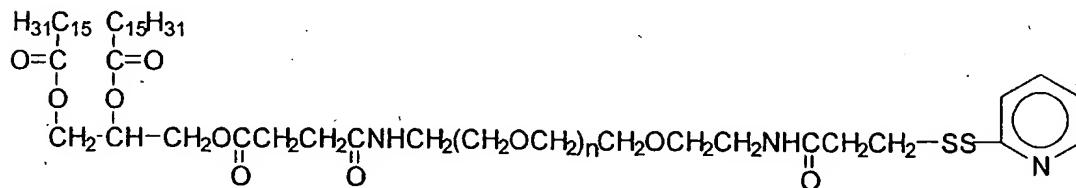
25 The various fragments will be resuspended at a concentration of 1 mg/mL in coupling buffer (150 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, 50 µm DTPA, pH 6.5) (all materials obtained from Sigma Chemical, St. Louis, MO). The lipid-PEG-maleimide lipid (0.2 to 10 mM) will then be added and the resulting mixture will be incubated for 16 hours at 4°C. The resulting products will be analyzed on a polyacrylamide electrophoresis gel to determine whether the Fab' and/or F(ab')₂ fragments became bound to the lipid. This

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can be determined by observing changes in molecular weight. Targeted vesicles will then be prepared using 1 wt % of the Fab' and/or F(ab')₂ bound lipids.

Example 47

- This example is directed to the preparation of DPGS-NH-PEG-NH-PDP,
 5 which has the following formula.

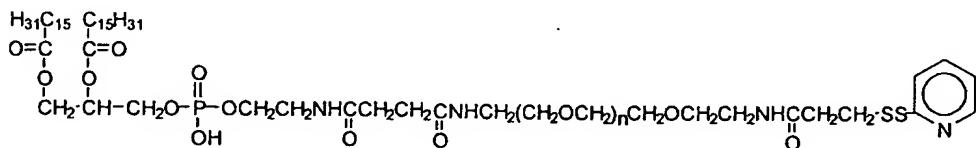


Dipalmitoyl glycerol succinate-polyethyleneglycol-amine

- (DPGS-NH-PEG-NH₂) was prepared utilizing the procedure in Example 44, step A.
 DPGS-NH-PEG-NH₂ (440 mg, 0.13 mmol) and triethylamine (13 mg, 0.13 mmol) were dissolved in CH₂Cl₂ (10 mL). To this solution was added a solution of
 10 3-(2-pyridylthio)propionic acid N-hydroxysuccinimide ester (PDP) (31 mg) in CH₂Cl₂ (10 mL). After stirring overnight at room temperature, deionized water (30 mL) was added to the reaction mixture and stirring was continued for an additional 3 hours. The lower organic layer was isolated using a separatory funnel and dried (Na₂SO₄). The organic layer was filtered and the filtrate was concentrated *in vacuo* to afford 360 mg of
 15 the title compound (DPGS-NH-PEG-NH-PDP) as a wax-like white solid.

Example 48

- This example is directed to the synthesis of DPPE-NH-PEG-NH-PDP,
 which has the following formula.

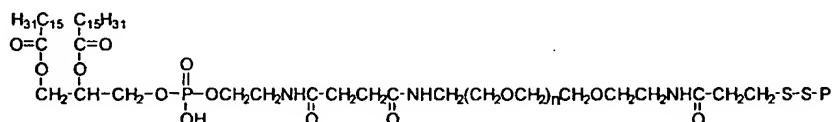


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1,2 Dipamitoyl-sn-glycerol-3-phosphoethanolamine-N-carbonylethylene-carbonyliminopolyethylene glycol ethylamine (DPPE-NH-PEG-NH₂) was prepared utilizing the procedure described in Example 45, Step A. This material (210 mg) and triethylamine (20 mg) were combined in CH₂Cl₂ (20 mL). This solution was added to a 5 solution of 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (PDP) (31 mg) in CH₂Cl₂ (10 mL). The resulting mixture was stirred for 1 hour at 0 to 5°C, and then stirred overnight at room temperature. Distilled water (10 mL) was added and the resulting mixture was acidified with dilute HCl, with stirring, to pH of 3. The organic layer was isolated, washed with water (10 mL) and dried (Na₂SO₄). The organic layer was 10 filtered and concentrated to dryness *in vacuo* using a rotary evaporator to provide 210 mg of the title compound (DPPE-NH-PEG-NH-PDP) as a white solid.

Example 49

This example is directed to the preparation of a lipid-hydrophilic polymer-protein conjugate within the scope of the present invention, which has the following 15 formula.



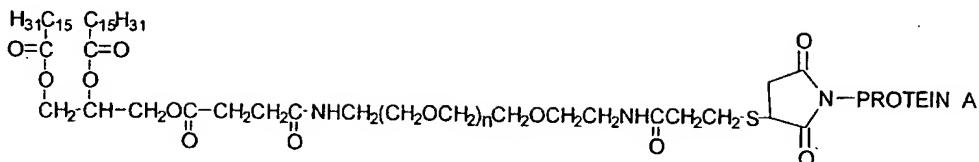
where "P" is—Protein A

The compound from Example 48 will be linked with Protein A by reacting a sulfur atom of the -S-S- disulfide bond in DPPE-NH-PEG-NH-PDP a sulphydryl group of a protein. The resulting compound may be utilized in forming protein-bearing vesicles, 20 such as protein-bearing liposomes.

Example 50

This example is directed to the preparation of a lipid-hydrophilic polymer-protein conjugate within the scope of the present invention, which has the following formula.

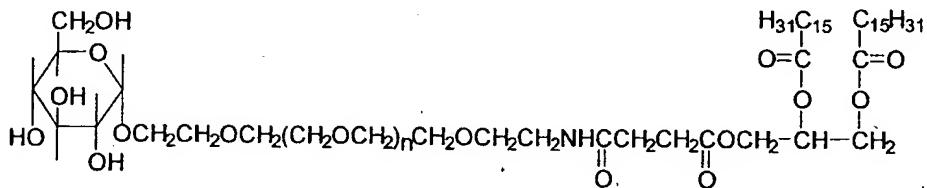
- 180 -



The compound from Example 47 (DPGS-NH-PEG-NH-PDP) will be coupled with maleimide-labeled Protein A to afford the above compound.

Example 51

- 5 This example is directed to the preparation of DPGS-NH-PEG-glucose, which has the following formula.



A. Preparation of NH_2 -PEG-Glucose.

- 2,3,4,6-tetra-O-Benzyl- α -D-glucopyranosyl bromide (0.6 g) was prepared according to the procedure of C.A.A. van Broeckel and J.H. van Boom, *Tetrahedron*, 41, 10 4545 (1985). Drying of the mixture was initiated by the use of activated molecular sieves in DMF mixed with ω -trifluoroacetylaminopolyethyleneglycol (3.5 g), the latter being prepared from ω -aminopolyethyleneglycol and triacetic acid anhydride, and diisopropylethylamine (DIEA) (1.2 mL). The resulting solution was stirred for 4 days under nitrogen. The solution was diluted with CHCl_3 (150 mL) and washed with 10% NaHCO_3 (50 mL) and water. The organic layer was concentrated *in vacuo*, and the resulting residue was treated with sodium carbonate methanol- H_2O solution at room temperature for 2 days. The solvent was removed *in vacuo* and the residue was extracted

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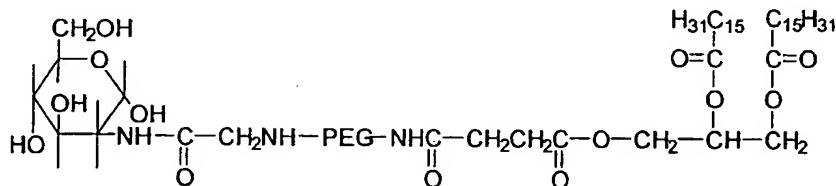
- twice with CHCl_3 (100 mL). The combined chloroform extracts were concentrated *in vacuo*, and the resulting residue was dissolved in methanol and hydrogenated over 10% palladium on charcoal (0.45 g) at 4 atmospheres of pressure and room temperature. The catalyst was filtered off and the filtrate was concentrated *in vacuo* to provide
- 5 $\text{NH}_2\text{-PEG-Glucose}$ (3 g) as a white solid. The product was purified on a silica gel column and eluted with an isocratic eluent of chloroform-methanol-water.

B. Preparation of DPGS-NH-PEG-Glucose.

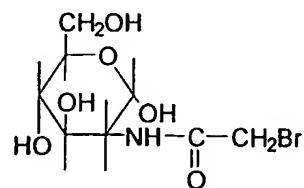
- $\text{NH}_2\text{-PEG-Glucose}$ from Step A (1 g), triethylamine (0.5 g) and a solution of acetonitrile and water (50:50) were combined and added to a cooled (0-5°C) solution of
- 10 DPGS-succinimide from Example 13A (0.15 g) in acetonitrile (10 mL). The resulting mixture was stirred at room temperature for two days and the acetonitrile was evaporated. The resulting residue was diluted with water to 50 mL, dialyzed through a 500 MWCO membrane, and lyophilized to yield the title compound (DPGS-NH-PEG-Glucose) as a white solid.

15 **Example 52**

This example is directed to the preparation of DPGS-NH-PEG-NH-Mannose, which has the following formula.



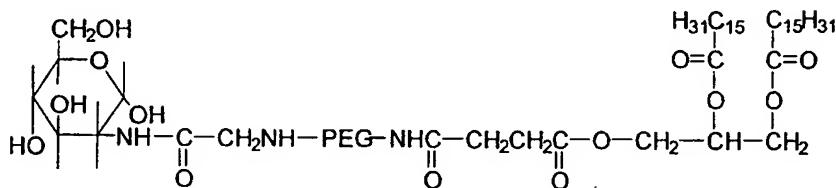
A. Preparation of α -bromo-acetylaminod-D-mannose.



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- D-Mannosamine hydrochloride (2.1 g), triethylamine (3 g) and DMF (50 mL) were combined and added to a cooled (0-5°C) solution of α -bromoacetyl bromide (2 g) in CH_2Cl_2 with stirring. The resulting mixture was stirred at room temperature for 8 hours, and then poured into ice water (50 mL). This aqueous mixture was stirred for 1 hour and then concentrated *in vacuo* to dryness. The resulting residue was recrystallized from a mixture of water and ethanol to afford α -bromo-acetylamino-D-mannose as a white solid (2.1 g).

B. Preparation of DPGS-NH-PEG-NH-Mannose.



- α -Bromoacetylamino-D-mannose from Step A (0.3 g),
- 10 DPGS-NH-PEG-NH₂ from Example 44A (4 g), sodium carbonate (0.2 g) and KI (20 mg) were combined in a mixture of water and ethanol (1:1). The resulting mixture was heated to 40°C for 8 hours. The ethanol was evaporated on a rotary evaporator, and the aqueous residue was dialyzed through a 500 MWCO membrane and lyophilized to yield 4 g of the title compound (DPGS-NH-PEG-NH-Mannose) as a white solid.

15 The following examples are directed to the preparation of targeted vesicles.

Example 53

- Albumin vesicles (or albumin coated gas bubbles) were suspended in a buffer solution with a pH greater than 8. To this suspension was added 3-(2-pyridyl)dithiopropionic acid N-hydroxy-succinimide ester (SPDP) in CH_3CN at 0 to 20 5°C. The resulting mixture was incubated for 2 days at room temperature and dialyzed on a membrane of 500 MWCO to provide pyridyldithiopropionoyl bearing albumin vesicles.

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Maleimidophenylbutyrate conjugated antibody (MPB-AB) was then incubated with the surface modified albumin vesicles to obtain antibody linked to albumin vesicles.

Example 54

Polyglutamic acid vesicles (or polyglutamic acid coated-gas bubbles) were suspended in water. To this suspension was added ethyl-N,N-dimethylaminopropylcarbodiimide hydrochloride (EDC) in water at 0 to 5°C. The resulting mixture was gently stirred for 4 hours and then a peptide solution (in a buffer of pH of 8 to 9.5) was added. This mixture was incubated for 2 days at room temperature and dialyzed on a membrane of 500 MWCO to afford peptide conjugated polyglutamic acid vesicles.

10 Example 55

Example 53 was repeated, except that vesicles formulated from a copolymer of methyl cyanomethacrylate (2-cyano-2-propenoic acid, methyl ester) and ω -amino-tetraethyleneglycolyl methacrylate were substituted for the albumin vesicles.

Example 56

15 This example is directed to the preparation of a lipid-polymer-peptide conjugate and targeted vesicles therefrom.

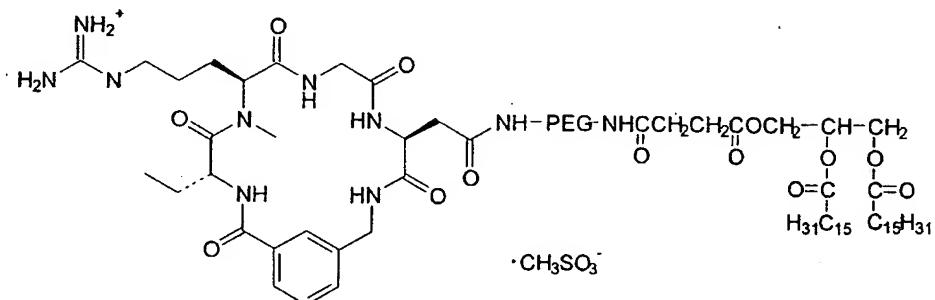
Into a round bottom flask was introduced 1 equivalent of α -amino, ω -carboxy PEG-4000 having a tert-butyloxycarbonyl (t-Boc) protecting group. This was activated by the addition of carbonyldiimidazole (CDI) (1 eq.) and hydroxybenzotriazole (1 eq.) In N-methylpyrrolidone. To this solution was added the peptide Lys-Gln-Ala-Gly-Asp-Val deprotected on the amino terminus. The resulting solution was stirred and periodically checked for free amino groups by analysis with methylene blue or ninhydrin. When there was no further evidence of the free amino terminus, deprotection of the entire mixture was carried out via the addition of trifluoroacetic acid in methylene chloride. Free amine from the PEG was then isolated by standard methods. Into a separate flask was introduced a solution of DPPE (1 eq) in DMF and carbonyldiimidazole (1 eq). Stirring was continued for one hour over molecular sieves (4 angstroms). To this mixture was then added the PEG-peptide ligand mixture followed by continued stirring. The mixture was

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then dried *in vacuo* and added to a DEAE Sepharose size exclusion column, thereby isolating the DPPE-PEG-peptide conjugate. Gas filled vesicles were then prepared from the DPPE-PEG-peptide utilizing standard methodology.

Example 57

- 5 This example is directed to the preparation of DPGS-PEG-cyclic peptide, which has the following formula.



Cyclic[D-2-aminobutyryl-N-2-methyl-L-arginyl-glycyl-L-aspartyl-3-(aminomethylbenzoic acid)] was prepared using standard peptide synthetic methodology.

- 10 To a solution of 66 mg of this cyclic peptide in water (20 mL) was added ethyl-N,N-dimethylaminopropylcarbodiimide hydrochloride (EDC) in water (10 mL) at 0 to 5°C. The resulting mixture was stirred for 4 hours at 0 to 5°C, followed by 8 hours at room temperature. To this solution was added DPGS-NH-PEG-NH₂ from Example 44A (400 mg) in CH₃CN (10 mL) at 0 to 5°C. After stirring overnight at room temperature, the
15 reaction mixture was concentrated *in vacuo* and the aqueous residue was dialyzed through a 1000 MWCO membrane. Lyophilizing provided the above compound (410 mg) as a white solid.

Example 58

- This example is directed to the preparation of vesicles targeted with a cyclic
20 peptide.

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To a solution of cyclic[D-2-aminobutyryl-N-2-methyl-L-arginyl-glycyl-L-aspartyl-3-(aminomethylbenzoic acid)] (66 mg) in water (20 mL) was added ethyl-N,N-dimethylaminopropylcarbodiimide hydrochloride (EDC) in water (10 mL) at 0 to 5°C. The resulting mixture was stirred for 4 hours at 0 to 5°C, followed by 8 hours at room temperature. The reaction mixture was then added to a suspension of albumin vesicles at 0 to 5°C. The resulting mixture was incubated for two days at room temperature, filtered and dialyzed using a membrane having a MWCO of 1000 to provide albumin vesicles targeted with a porphyrin targeting ligand.

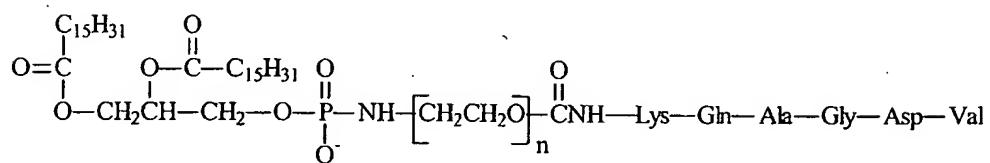
Example 59

10 Example 58 was repeated, except that vesicles formulated from a copolymer of methyl cyanomethacrylate (2-cyano-2-propenoic acid, methyl ester) and ω -amino-tetraethyleneglycol methacrylate were substituted for the albumin vesicles.

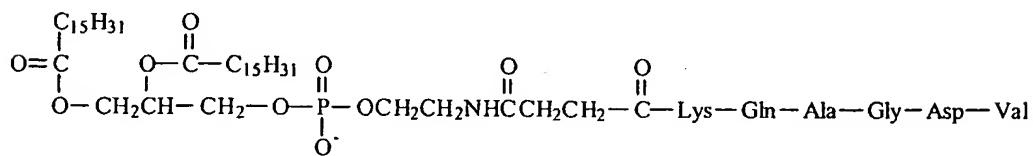
Example 60

15 Using the synthetic methodologies described above, the following compounds were prepared.

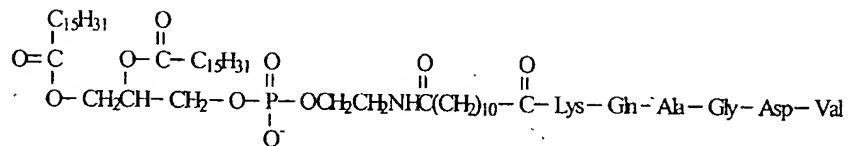
A. DPPA-PEG-KQAGDV, which has the formula



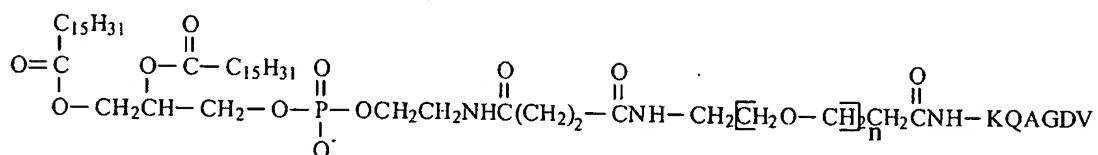
B. DPPE-succinyl-KQAGDV, which has the formula



C. DPPE-dodecanyl-PEG-KQAGDV which has the formula

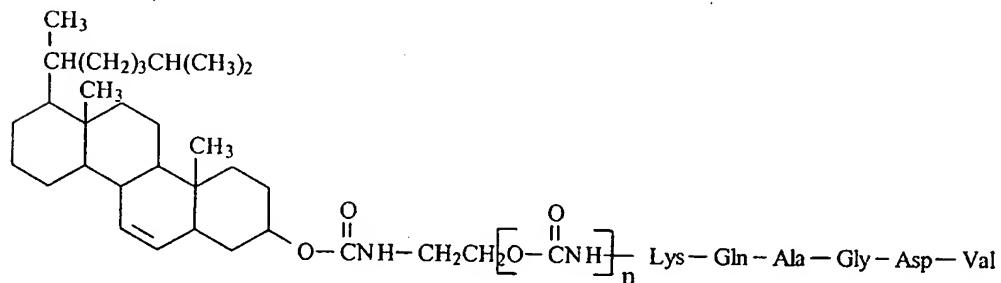


D. DPPE-succinyl-PEG-KQAGDV, which has the formula

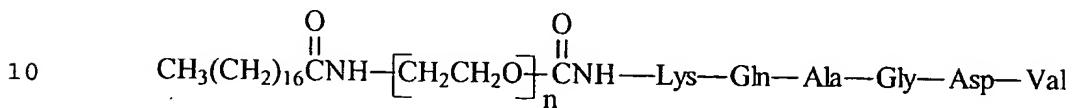


5 where KQAGD is Lys-Gln-Ala-Gly-Asp-Val

E. Cholesteryl-PEG-KQAGDV which has the formula



F. Stearyl-PEG-KQAGDV, which has the formula



Example 61

This example is directed to the preparation of targeted vesicles within the scope of the present invention.

The targeted lipid DPGS-PEG-KQAGDV (1.25 wt %) and the lipids DPPA 5 (6 wt % minus the weight of the DPGS-PEG-KQAGDV), DPPE-PEG5000 (40 wt % minus the weight of DPGS-PEG-KQAGDV) and DPPC (54 wt % minus the weight of DPGS-PEG-KQAGDV) were introduced into an evaporating flask. Aliquots of a mixture of toluene and methanol (60:40 v/v) were added to dissolve the lipids and rinse the lipids into the flask. The flask was placed on a rotary evaporator and warmed to 55°C ± 5°C until 10 the solution in the flask was clear. Vacuum was applied to the rotary evaporator to concentrate the solution to provide a thick gel. The flask was then placed on a magnetic stir plate and a stir bar was placed in the flask. Methyl t-butyl ether (MTBE) was added to the flask to precipitate solid material, and the solid mixture was cooled to 25°C ± 5°C. The resulting white solid was collected via vacuum filtration, washed with MTBE and placed 15 in a vacuum oven and dried for at least 4 hours to a constant weight at 45°C ± 5°C. Vesicles were prepared from the lipid mixture using the procedures discussed herein. When not in use, the lipid mixture was stored in an airtight container at -15°C ± 5°C.

Example 62

This example includes a description of comparative testing of the targeted 20 contrast agents of the present invention to contrast agents of the prior art.

Testing System

A testing system of the type depicted in Figure 1 was employed in this example. Polyethylene tubing (Pharmed, Cole-Parmer) was passed through a block composed of 2% agar osc. The agar was block prepared by heating the agar in water and 25 pouring it into a plastic mold (dimensions = 8 cm x 10 cm). The agar osc was purchased from Behringer Mannheim (Indianapolis, Indiana). Peristaltic flow was provided by a Master-Flex, Cole-Parmer pump. Human blood was drawn from normal male volunteers and the blood was allowed to clot on cotton piping material and fastened to #6 gauge

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fishhooks, which were attached to nylon filament. The nylon filament was threaded through the polyethylene tubing clots such that the clots were positioned in the center of the tubing within the agar blocks. Contrast agents were injected upstream of the clot and the pump was used to circulate saline past the clot at a rate of 50 cc per ml for 30 seconds

5 which was increased to a rate of 150 cc per minute thereafter. Ultrasound imaging was performed with a Sonos model No. 5500 Hewlett Packard clinical ultrasound machine using a linear array second harmonic transducer (2 to 4 MHz) operating in the fundamental mode. Ultrasound was performed continuously at a mechanical index of 0.2 with the focal zone of the transducer directed on the clot. A region of interest was drawn on the clots and

10 signal intensity was measured before injection of the contrast agents and for 2 and up to 5 minutes after injection of the contrast agent.

Contrast Agents Employed in Comparative Testing

A stock lipid mix was prepared from 82 weight % dipalmitoylphosphatidyl-choline, 8 weight % dipalmitoylphosphatidic acid and 10 weight % dipalmitoyl-phosphatidylethanolamine-PEG 5000. To aliquots of this stock lipid mix were added varying weight percents ranging from 0.05 weight % to 5 weight %, based on the weight of dry lipid employed in the stock lipid mix, of the targeted lipids DPPE-PEG-KQAGDV, cholesterol-PEG-KQAGDV, stearyl-PEG-KQAGDV, dipalmitoylphosphatidylglycerol-PEG-KQAGDV and unbound or free peptide KQAGDV. The molecular weight of the

15 PEG in the targeted lipids DPPE-PEG-KQAGDV, cholesterol-PEG-KQAGDV, stearyl-PEG-KQAGDV and dipalmitoylphosphatidylglycerol-PEG-KQAGDV was MW 3400.

The lipid mix with targeted lipid or free peptide was then suspended in a solution comprised of 8:1:1 v:v:v normal saline, propylene glycol and glycerol at a final lipid concentration of 1 mg/mL, and 1.5 mL of each sample was then added to 2 mL clear glass

20 vials. The vials were capped and the head-space of the vials evacuated and exchanged with perfluorobutane gas. The vials were then shaken on a modified dental amalgamator at 4,200 rpm for 60 seconds (ESPE, Secfeld, Germany) resulting in phospholipid coated microbubbles.

25

The size and concentration of the microbubbles was determined with an

30 Accusizer Model No. 770 particle sizer (Particle Sizing Systems, Goleta, California). All

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of the different preparations of gas filled vesicles had similar size with mean diameter of about 2 microns and particle concentration of about 1.5 billion bubbles per mL.

Samples (1 mL volume) of the various targeted vesicle compositions were injected upstream of the blood clot via an injection port and the pump was circulated at low and high speed. The ultrasound intensity of the clots was measured for the different compositions. A composition containing non-targeted vesicles, prepared from the stock solution, did not cause any appreciable increase in signal intensity of the clots. The ultrasound signal intensity obtained from this composition is depicted graphically in Figure 2. In contrast, all of the vesicles containing the different targeted lipids increased the signal intensity of the clots. Figure 3 includes a graphical depiction of the signal intensity of the blood clot before and after exposure to vesicles prepared from the stock lipid solution and 0.3% by weight of the lipid conjugate DPPE-PEG-KQAGDV. An increased signal intensity was surprisingly obtained from a vesicle composition prepared from the stock vesicle solution and 0.3% by weight unbound or free KQAGDV. This increase in ultrasound intensity is depicted graphically in Figure 4. This study demonstrates that the composition containing the free peptide was at least as effective as the composition containing the lipid conjugate DPPE-PEG-KQAGDV.

Example 63

A targeted vesicle composition comprising perfluorobutane gas microbubbles encapsulated by lipid vesicles formulated from dipalmitoylphosphatidyl-choline, dipalmitoylphosphatidic acid, dipalmitoylphosphatidylethanolamine-PEG 5000 and dipalmitoyl-sn-glycero-3-succinyl-PEG-Lys-Gln-Ala-Gly-Asp-Val was prepared as described herein. Approximately 0.05 mL of a non-targeted vesicle composition comprising perfluoropropane gas microbubbles encapsulated by lipid vesicles formulated from dipalmitoylphosphatidylcholine, dipalmitoylphosphatidic acid, dipalmitoyl-phosphatidylethanolamine-PEG 5000 and 0.05 mL of the aforementioned targeted vesicle composition were each incubated with separate samples of fresh whole human blood clot prepared from coagulation of whole human blood (each sample having a volume of about 1.0 mL). After one minute, the clots were washed 8 times with 10 mL of normal saline to remove unbound vesicles. The vesicles were then insonated at 2 MHz using a Panametrics

transducer. A 5 MHz transducer was used to receive backscattered echoes from the clot. The signal was displayed on a LeCroy oscilloscope. The signals were received and analyzed on a PC where a Fourier transform was performed to produce the spectra as shown in Figure 7. The fundamental insonation frequency was 2.25 MHz. Multiple 5 harmonics are visible at 4.5, 6.75, 9 and 11.25 MHz, i.e., at 2x, 3x, 4x and 5x, respectively. As shown in Figure 7, the harmonic signatures of the targeted vesicle composition may be much greater than the signals for either the native clot or clot in combination with the non-targeted vesicle composition.

Example 64

10 Human clot is again prepared and the targeted vesicle composition from microbubble agent described in Example 63 is incubated with the clot. Fundamental imaging is performed with a 3.0 MHz signal using an Acoustic Imaging Model 5200 ultrasound scanner. Faint enhancement of the clot is seen on the fundamental images. Subsequently, the clot is insonated simultaneously at 1.0 MHz while a 7.5 MHz clinical 15 transducer is also employed. Significant enhancement was attained when the two frequencies were employed as compared to a single frequency.

Example 65

Fresh artificial clots were made from citrated whole blood using Chandler's loop method. Individual clots were placed in a 20 mL chamber immersed in a 37°C water 20 bath and random selections were made to be treated with any of the following:

- A. PBS (control);
 - B. Targeted vesicle composition with/without urokinase (UK) with/without external ultrasound (US);
 - C. UK with/without non-targeted vesicle composition with/without US.
- 25 Continuous 20 kHz and 1 MHz ultrasound were used with alternating 2 seconds on and off for 5 minutes. The peak acoustic pressure was 300 kilopascals for 1 Mhz and 0.9 kilopascals for 20 kHz. The temperature variation in the chamber was maintained within 2°C.

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Treated clots were incubated for 30 minutes and left in Bouin's solution overnight. The clots were washed with PBS, dried and weighed. Lysis efficiency was determined by the relative clot weight loss compared to the control group.

Example 66

5 The procedure of Example 65 is repeated, except with 20 kHz and 1 MHz used simultaneously.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

Various modification of the invention, in addition to those described herein,
10 will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

CLAIMS**What is claimed is:**

1. An ultrasound method comprising (i) administering to a patient a targeted vesicle composition which comprises vesicles comprising a lipid, protein or polymer, encapsulating a gas, in combination with a targeting ligand; and (ii) scanning the patient using ultrasound, wherein said scanning comprises exposing the patient to a first type of ultrasound energy and then interrogating the patient using a second type of ultrasound energy.
2. A method according to Claim 1, wherein the method is used for detection of a thrombus.
3. A method according to Claim 2, wherein said thrombus comprises an old or echogenic thrombus.
4. A method according to Claim 1, wherein the method is used for detecting low concentrations of vesicles.
- 15 5. A method according to Claim 1, wherein the method is used for detecting vesicles targeted to endothelial tissue.
6. A method according to Claim 5, wherein said endothelial tissue includes integrins associated with malignancy or inflammation in early or small lesions.
- 20 7. A method according to Claim 1, comprising the use of insonation frequencies with a bandwidth of about 100 kHz or less.
8. A method according to Claim 7, wherein said bandwidth is about 50 kHz or less.

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9. A method according to Claim 8, wherein said bandwidth is about 10 kHz or less.

10. A method according to Claim 1, comprising the use of a center insonation frequency with a bandwidth of less than about 1 kHz.

5 11. A method according to Claim 1, wherein the method comprises the use of a broadband receiver and a digital filter with multiple center frequencies, wherein said center frequencies are digitally adjusted with respect to an insonating frequency, and filter gates and bandwidth windows are selected and controlled such that said filter rejects signals with center frequencies outside of a selected range.

10 12. A method according to Claim 1, comprising the use of pulsing and phase modulation of said first and second types of ultrasonic energy.

13. A method according to Claim 1, wherein said first and second types of ultrasonic energy comprise pulses with different insonation frequencies.

14. A method according to Claim 13, comprising first insonating at a
15 lower frequency in a range of from about 20 kHz to about 10 MHz, and then insonating at a higher frequency in a range of from about 20 kHz to about 50 MHz.

15. A method according to Claim 14, wherein said lower frequency is about 1 MHz and said higher frequency is about 3 MHz.

16. A method according to Claim 14, wherein said higher frequency is
20 at least two times said lower frequency.

17. A method according to Claim 14, wherein said lower frequency is about 100 kHz and said higher frequency is about 3 MHz.

18. A method according to Claim 14, wherein said lower frequency is administered as a pulse train.

19. A method according to Claim 18, wherein said pulse train comprises about 10 or fewer pulses.

5 20. A method according to Claim 14, wherein said lower insonation frequency is administered with a single pulse of low frequency sound, and is followed by pulses of higher frequency sound.

10 21. A method according to Claim 14, wherein a single pulse of about 100 kHz is applied and within about 40 milliseconds one or several pulses of higher frequency ultrasound are applied.

22. A method according to Claim 1, comprising forming a summation of several pulses given in rapid succession after an initial stimulation pulse, whereby the signal-to-noise ratio is improved.

15 23. A method according to Claim 1, wherein said first and second types of ultrasound energy comprise pulses that are phase modulated or delayed with respect to each other.

24. A method according to Claim 23, wherein said phase modulation and/or time delay is/are selected to maximize vesicle oscillation and collapse.

20 25. A method according to Claim 23, wherein said first type of ultrasound energy comprises one pulse or a pulse train with a duration from about 10 microseconds to about 10 seconds.

26. A method according to Claim 25, wherein said duration is from about 1 millisecond to about 2 seconds.

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27. A method according to Claim 22, wherein a peak acoustic pressure for a first pulse of about 100 pascals to about 10 megapascals is employed.

28. A method according to Claim 27, wherein said peak acoustic pressure is about 1 kilopascal to about 5 megalapascals.

5 29. A method according to Claim 28, wherein said peak acoustic pressure is about 10 kilopascals to about 5 megapascals.

30. A method according to Claim 1 wherein said vesicles comprise lipid vesicles.

31. A method according to Claim 30 wherein said lipid vesicles are
10 selected from the group consisting of micelles and liposomes.

32. A method according to Claim 30 wherein said lipid comprises a phospholipid.

33. A method according to Claim 32 wherein said phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine and
15 phosphatidic acid.

34. A method according to Claim 33 wherein said phosphatidylcholine is selected from the group consisting of dioleoylphosphatidylcholine, dimyristoylphosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

20 35. A method according to Claim 34 wherein said phosphatidylcholine comprises dipalmitoylphosphatidylcholine.

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36. A method according to Claim 34 wherein said phosphatidylethanolamine is selected from the group consisting of dipalmitoylphosphatidylethanolamine, dioleoylphosphatidylethanolamine, N-succinyldioleoylphosphatidylethanolamine and 1-hexadecyl-2-palmitoylglycerophosphoethanolamine.

5 37. A method according to Claim 36 wherein said phosphatidylethanolamine comprises dipalmitoylphosphatidylethanolamine.

38. A method according to Claim 33 wherein said phosphatidic acid comprises dipalmitoylphosphatidic acid.

10 39. A method according to Claim 30 wherein said lipid further comprises a polymer.

40. A method according to Claim 39 wherein said polymer comprises a hydrophilic polymer.

41. A method according to Claim 40 wherein said polymer comprises polyethylene glycol.

15 42. A method according to Claim 1 wherein said vesicles comprise protein vesicles.

43. A method according to Claim 42 wherein said protein comprises albumin.

20 44. A method according to Claim 1 wherein said vesicles comprise polymer vesicles.

45. A method according to Claim 44 wherein said polymer comprises synthetic polymers or copolymers which are prepared from monomers selected from the

group consisting of acrylic acid, methacrylic acid, ethyleneimine, crotonic acid, acrylamide, ethyl acrylate, methyl methacrylate, 2-hydroxyethyl methacrylate, lactic acid, glycolic acid, ϵ -caprolactone, acrolein, cyanoacrylate, bisphenol A, epichlorhydrin, hydroxyalkylacrylates, siloxane, dimethylsiloxane, ethylene oxide, propylene oxide,

- 5 ethylene glycol, hydroxyalkylmethacrylates, N-substituted acrylamides, N-substituted methacrylamides, N-vinyl-2-pyrrolidone, 2,4-pentadiene-1-ol, vinyl acetate, acrylonitrile, styrene, p-amino-styrene, p-aminobenzylstyrene, sodium styrene sulfonate, sodium 2-sulfoxyethyl-methacrylate, vinyl pyridine, aminoethyl methacrylates and 2-methacryloyloxytrimethyl-ammonium chloride.

- 10 46. A method according to Claim 44 wherein said polymer comprises synthetic polymers or copolymers selected from the group consisting of polyacrylic acid, polyethyleneimine, polymethacrylic acid, polymethylmethacrylate, polysiloxane, polydimethylsiloxane, polylactic acid, poly(ϵ -caprolactone), epoxy resin, poly(ethylene oxide), poly(propylene oxide), poly(ethylene glycol), polyamide, polyvinylidene-
15 polyacrylonitrile, polyvinylidene-polyacrylonitrile-polymethyl-methacrylate and polystyrene-polyacrylonitrile.

47. A method according to Claim 46 wherein said polymer comprises polyvinylidene-polyacrylonitrile copolymer.

- 20 48. A method according to Claim 1 wherein said fluorinated gas comprises a perfluorocarbon.

49. A method according to Claim 48 wherein said perfluorocarbon gas is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane and perfluorocyclobutane.

50. A method according to Claim 49 wherein said perfluorocarbon gas
25 is selected from the group consisting of perfluoropropane and perfluorobutane.

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51. A method according to Claim 50 wherein said perfluorocarbon gas comprises perfluorobutane.

52. A method according to Claim 1 wherein said gas is derived, at least in part, from a gaseous precursor.

5 53. A method according to Claim 52 wherein said gaseous precursor has a boiling point of greater than about 37°C.

54. A method according to Claim 52 wherein said gaseous precursor comprises a perfluorocarbon.

55. A method according to Claim 54 wherein said perfluorocarbon is
10 selected from the group consisting of perfluoropentane and perfluorohexane.

56. A method according to Claim 1 wherein said targeting ligand targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein GPIIbIIIa receptor.

57. A method according to Claim 56 wherein said targeting ligand is
15 selected from the group consisting of proteins, peptides, saccharides, steroids, steroid analogs, bioactive agents and genetic material.

58. A method according to Claim 57 wherein said targeting ligand is selected from the group consisting of proteins, peptides and saccharides.

59. A method according to Claim 58 wherein said targeting ligand is
20 selected from the group consisting of proteins and peptides.

60. A method according to Claim 59 wherein said targeting ligand comprises a peptide.

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61. A method according to Claim 60 wherein said peptide comprises a sequence selected from the group consisting of Arg-Gly-Asp and Lys-Gln-Ala-Gly-Asp-Val.

62. A method according to Claim 1 wherein said targeting ligand is
5 associated with said lipid, protein or polymer covalently.

63. A method according to Claim 62 wherein said targeting ligand is unbound.

64. A method according to Claim 63 wherein said targeting ligand is associated with said lipid, protein or polymer non-covalently.

10 65. A method according to Claim 62 wherein said covalent association comprises a covalent bond selected from the group consisting of amide, thioamide, ether, ester, thioester, -O-, -S-, -S_n-, where n is greater than 1, carbamate, -NH-, -NR-, where R is alkyl of from 1 to about 4 carbons, urethane, and substituted imidate bonds.

15 66. A method according to Claim 65 wherein said covalent association further comprises crosslinking.

67. A method according to Claim 62 wherein said targeting ligand is covalently associated with said lipid, protein or polymer via a linking group.

68. A method according to Claim 67 wherein said linking group comprises a hydrophilic polymer.

20 69. A method according to Claim 68 wherein said hydrophilic polymer is selected from the group consisting of polyalkyleneoxides, polyvinyl alcohol, polyvinylpyrrolidones, polyacrylamides, polymethacrylamides, polyphosphazenes, poly(hydroxylalkylcarboxylic acids) and polyoxazolidines.

- 200 -

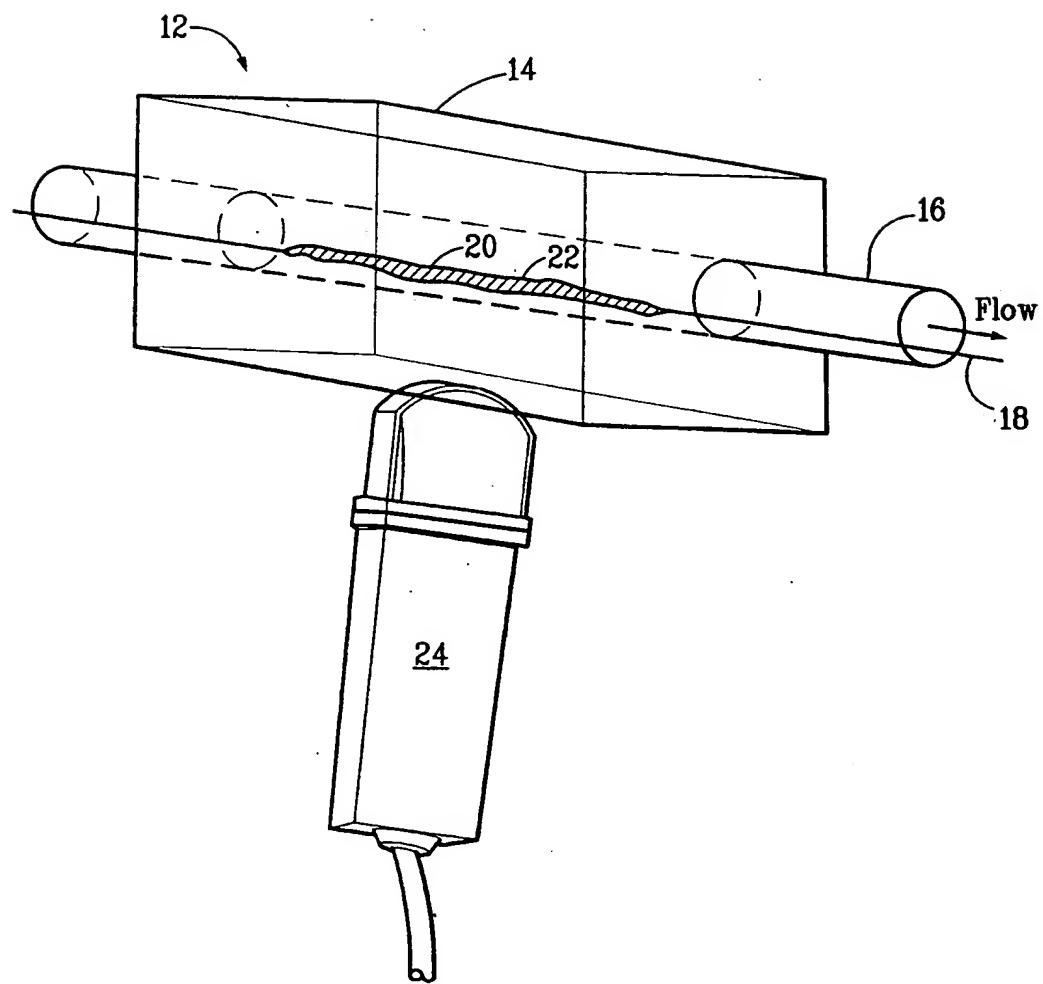
70. A method according to Claim 69 wherein said hydrophilic polymer comprises a polyalkyleneoxide.

71. A method according to Claim 70 wherein said polyalkyleneoxide is selected from the group consisting of polyethylene glycol and polypropylene glycol.

5

72. A method according to Claim 71 wherein said polyalkyleneoxide comprises polyethylene glycol.)

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*FIG. 1*10

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FIG. 2

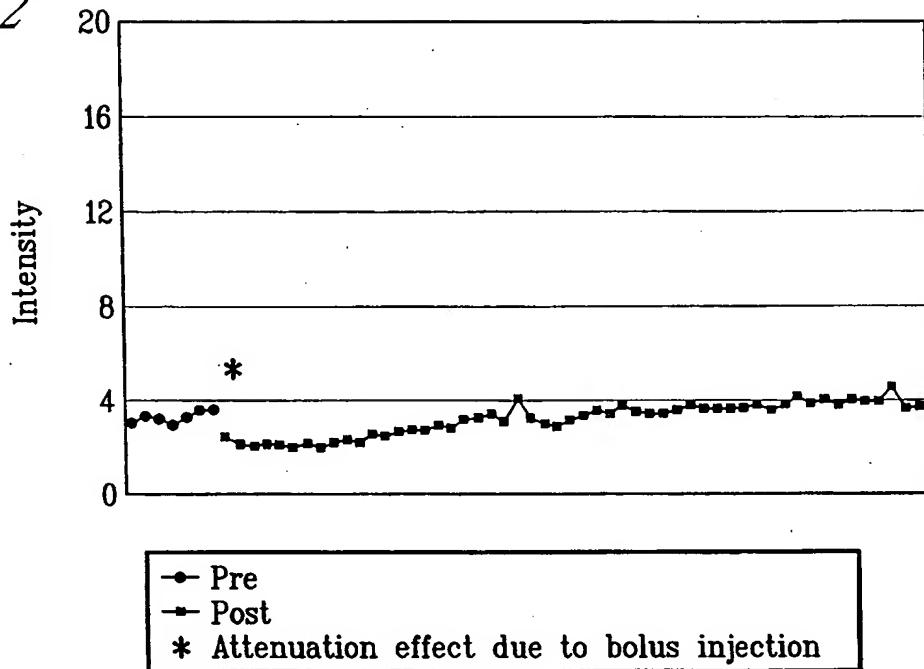
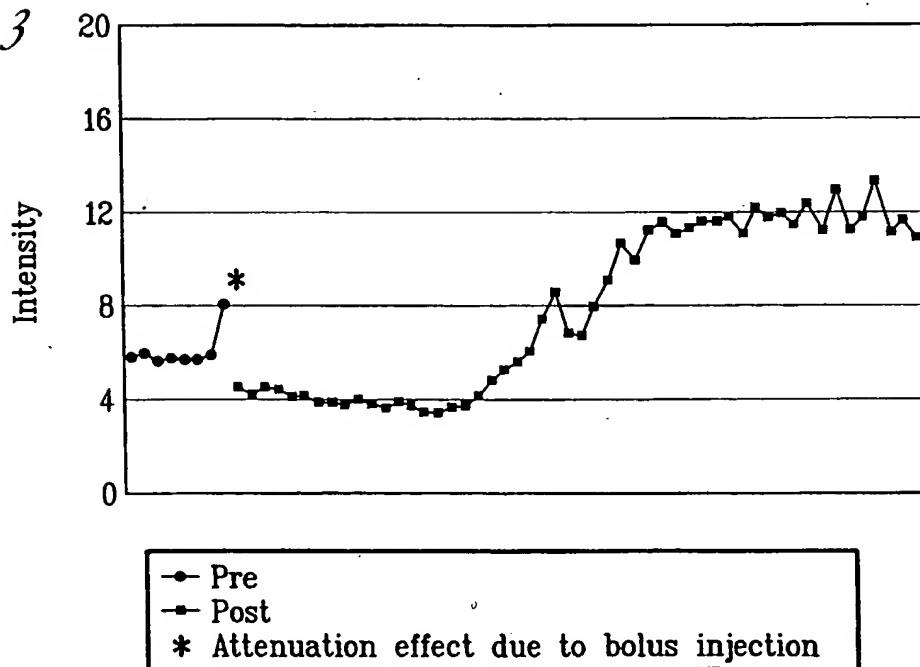


FIG. 3



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FIG. 4

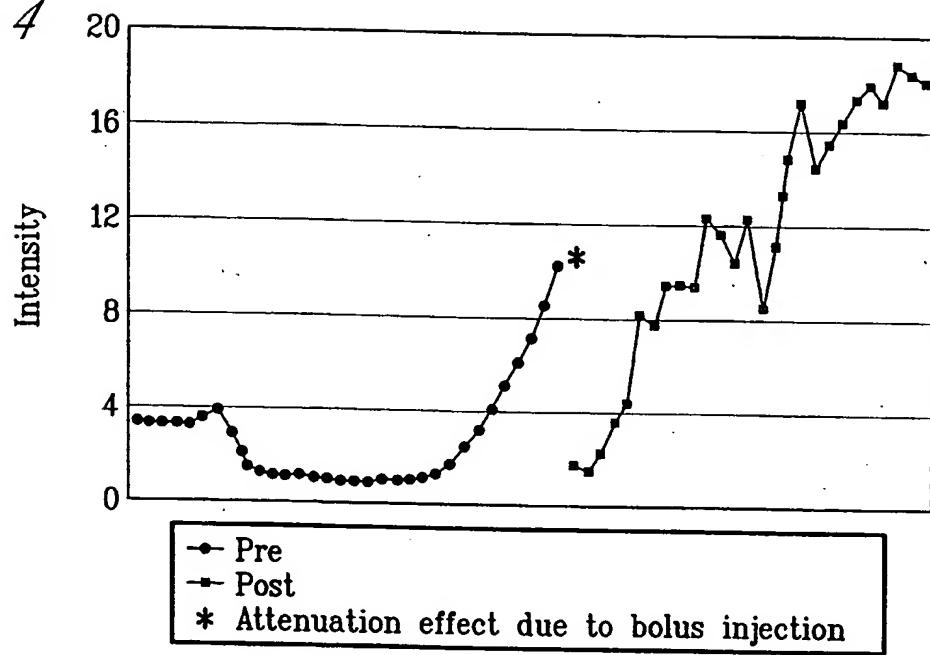


FIG. 5

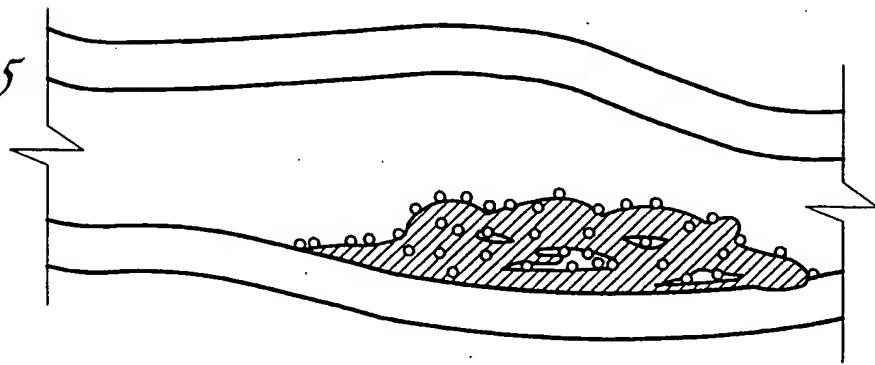
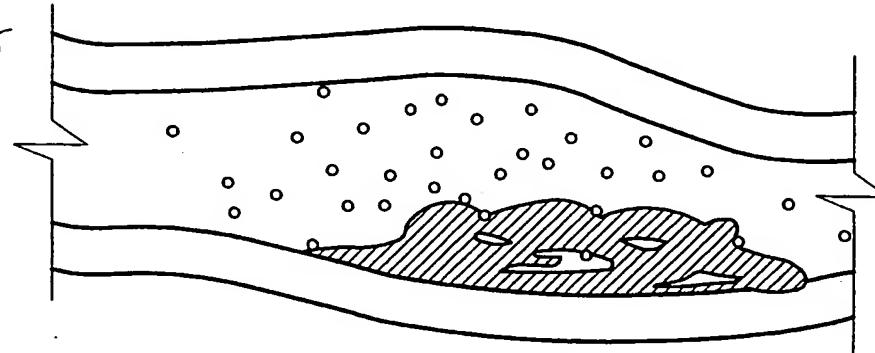


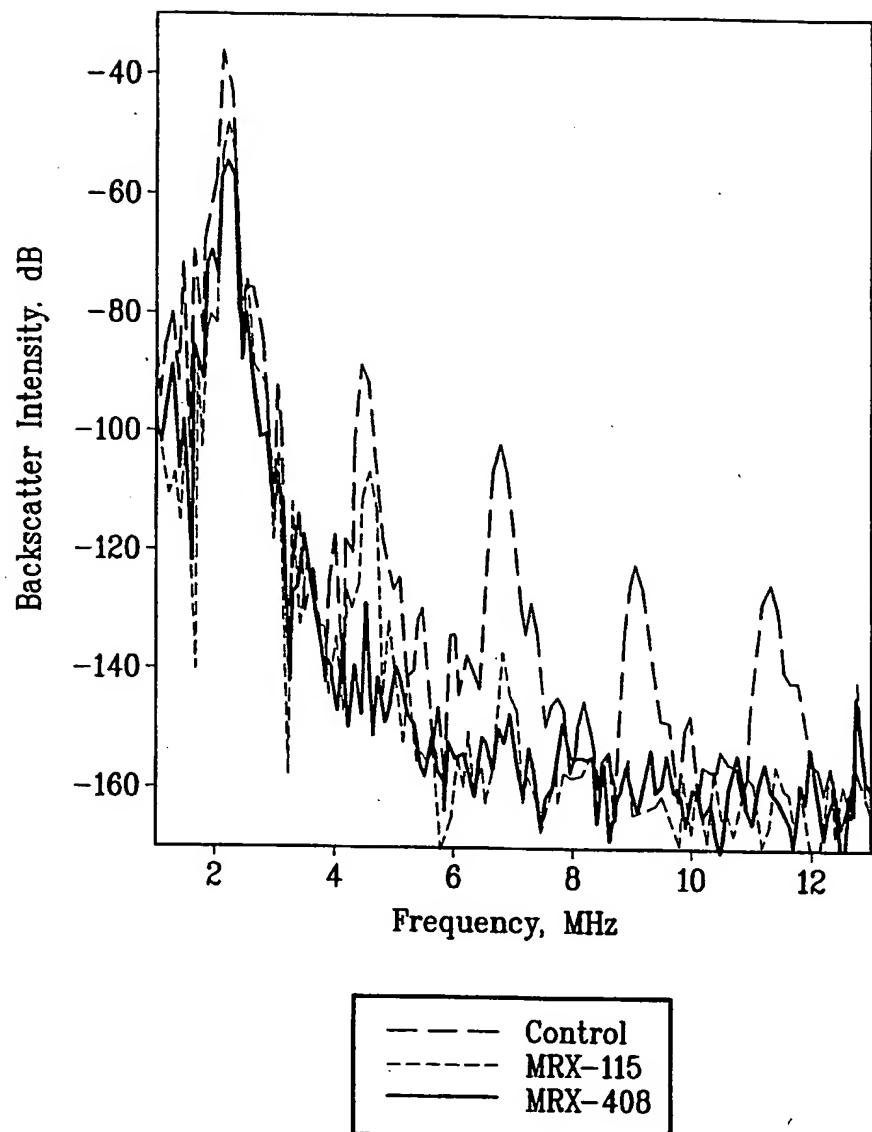
FIG. 6



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FIG. 7

Backscatter of Blood Clot B



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FIG. 8

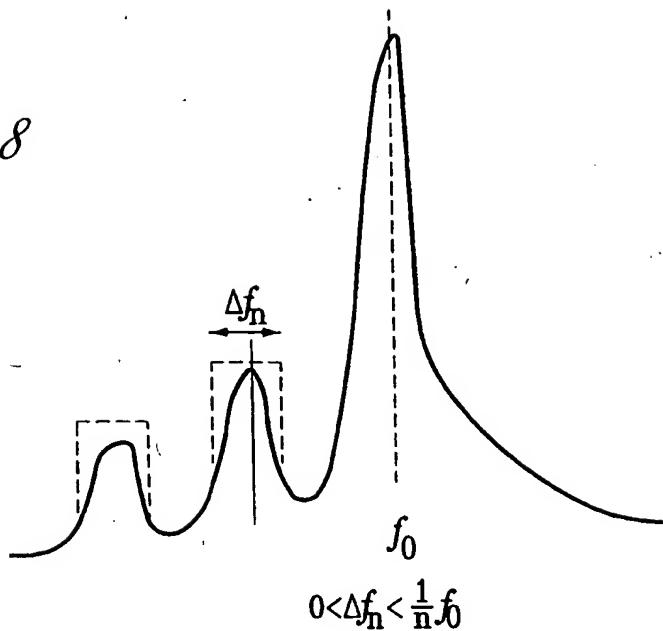
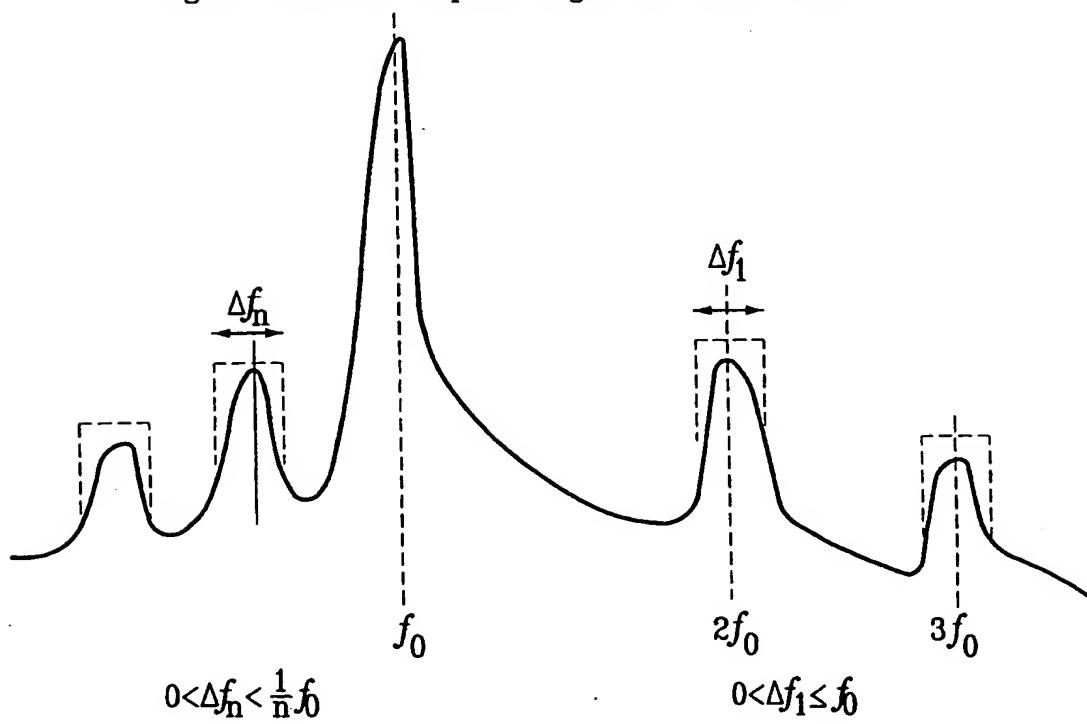


FIG. 9

Digital filters to improve signal to noise ratio



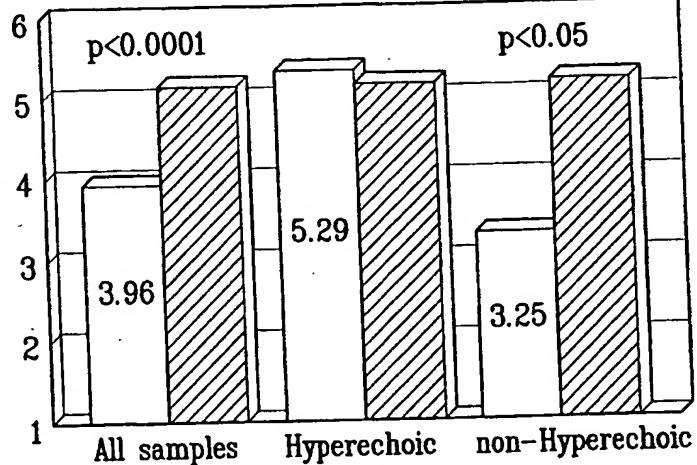
6/9

FIG. 10



Results of Review for Presence of Thrombus

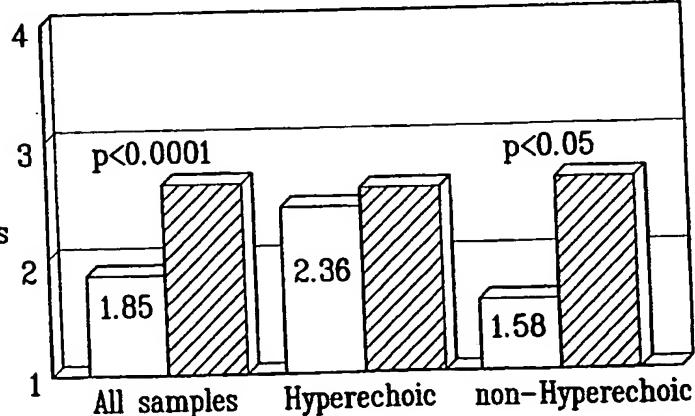
	Pre	Post
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Scoring Scale
 1 = Definitely not present
 2 = Probably not present
 3 = Possibly not present
 4 = Possibly present
 5 = Probably present
 6 = Definitely present

Results of Review for Thrombus Enhancement

	Pre	Post
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Scoring Scale
 1 = No thrombus seen
 2 = Hypoechoic thrombus
 3 = Isoechoic thrombus
 4 = Hyperechoic thrombus

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FIG. 11A

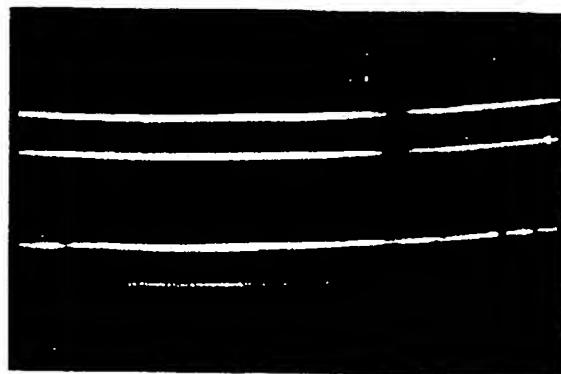


FIG. 11B

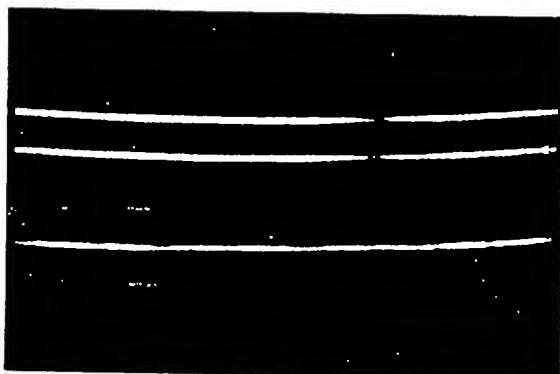
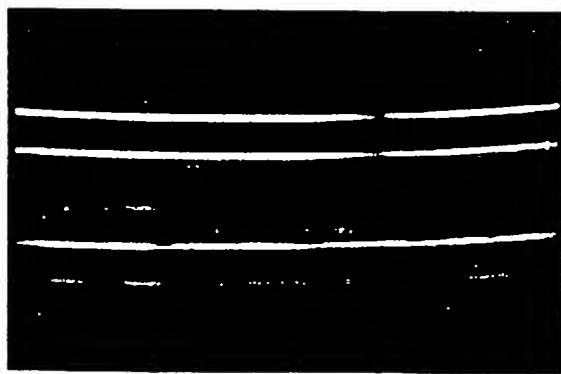


FIG. 11C



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FIG. 12

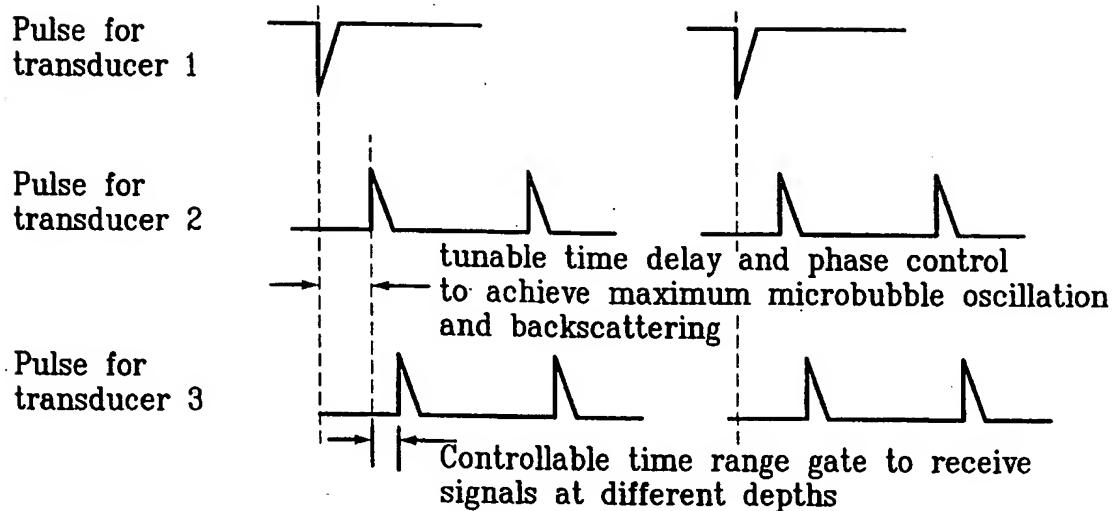
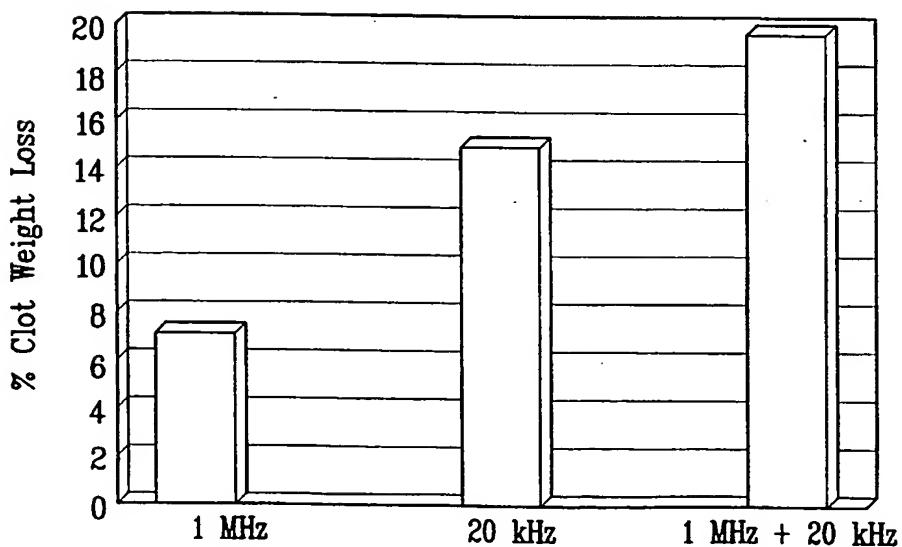
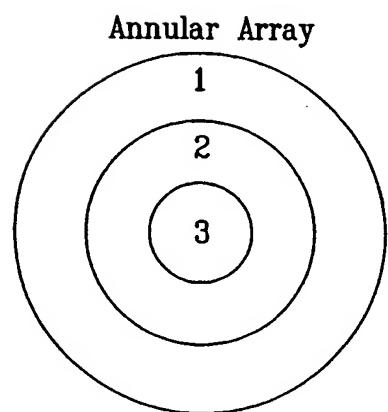
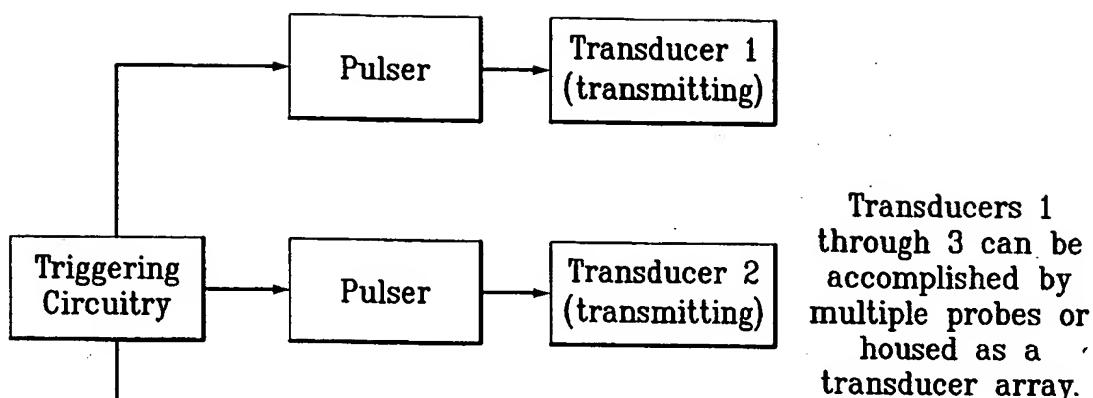


FIG. 13

Clot Lysis with Multiple Frequencies
(for 5 Minutes)
With MRX-408



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1 = Transmit
2 = Transmit
3 = Receive

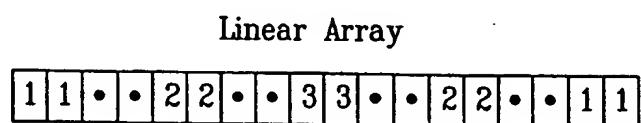


FIG. 14

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IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,
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- (54) Title: NOVEL METHODS OF IMAGING AND TREATMENT WITH TARGETED COMPOSITIONS

(57) Abstract: Novel ultrasound methods comprising administering to a patient a targeted vesicle composition which comprises vesicles comprising a lipid, protein or polymer, encapsulating a gas, in combination with a targeting ligand, and scanning the patient using ultrasound. The scanning may comprise exposing the patient to a first type of ultrasound energy and then interrogating the patient using a second type of ultrasound energy. The targeting ligand preferably targets tissues, cells or receptors, including myocardial cells, endothelial cells, epithelial cells, tumor cells and the glycoprotein GPIIbIIIa receptor. The methods may be used to detect a thrombus, enhancement of an old or echogenic thrombus low concentrations of vesicles and vesicles targeted to tissues, cells or receptors.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER IPC 7 A61K49/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) PAJ, WPI Data, EPO-Internal, BIOSIS, MEDLINE, CANCERLIT, EMBASE, DISSERTATION ABS CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,L	<p>WO 96 40285 A (IMARX PHARMACEUTICAL CORP ;UNGER EVAN C (US); SHEN DEKANG (US); WU) 19 December 1996 (1996-12-19) the whole document L: Priority</p> <p style="text-align: center;">—/—</p>	1-72
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		<input checked="" type="checkbox"/> Patent family members are listed in annex.
<p>* Special categories of cited documents :</p> <ul style="list-style-type: none"> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed 		
Date of the actual completion of the international search 18 October 2000		Date of mailing of the international search report 22/11/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer Dullaart, A

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 00/02620

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>UNGER EVAN C ET AL: "In vitro studies of a new thrombus-specific ultrasound contrast agent." <i>AMERICAN JOURNAL OF CARDIOLOGY</i>, vol. 81, no. 12 SUPPL. A, 12 June 1998 (1998-06-12), pages 58G-61G, XP002087505 & Symposium: Ninth International Congress on Echocardiography: Clinical Cardiology, 1997 ISSN: 0002-9149 abstract page 58G, right-hand column page 59G, right-hand column, paragraph RESULTS page 61G, left-hand column, paragraph DISCUSSION</p> <hr/> <p>WU YUNQIU ET AL: "Preliminary report: Binding and lysing of blood clots using MRX-408." <i>INVESTIGATIVE RADIOLOGY</i>, vol. 33, no. 12, December 1998 (1998-12), pages 880-885, XP000952676 ISSN: 0020-9996 abstract page 882, left-hand column figure 6 page 884, right-hand column, paragraph DISCUSSION -page 885</p> <hr/> <p>TAKEUCHI M ET AL: "Enhanced visualization of intravascular thrombus with the use of a thrombus targeting ultrasound contrast agent (MRX408): Evidence from in vivo experimental echocardiographic studies." <i>JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY</i>, vol. 31, no. 2 SUPPL. A, February 1998 (1998-02), page 57A, abstract 803-6 XP000952675 & 47th Annual Scientific Session of the American College of Cardiology; Atlanta, Georgia, USA; March 29-April 1, 1998 ISSN: 0735-1097 abstract</p> <hr/> <p style="text-align: center;">-/-</p>	1-72
Y		1-72
Y		1-72

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02620

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>HAUTANEN A ET AL: "Effects of modifications of the RGD sequence and its context on recognition by the fibronectin receptor."</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, JAN 25 1989, VOL. 264, NO. 3, PAGE(S) 1437-1442, XP002150374</p> <p>abstract</p> <p>page 1439, left-hand column, last paragraph -right-hand column, line 15 figure 5</p> <p>page 1441, right-hand column, line 39 - line 56</p> <p>-----</p>	1-72
P,X, L	<p>EP 0 901 793 A (IMARX PHARMACEUTICAL CORP) 17 March 1999 (1999-03-17)</p> <p>paragraph '0190!</p> <p>L: Priority</p> <p>example 3</p> <p>-----</p>	1-72
P,X, L	<p>WO 99 13919 A (IMARX PHARMACEUTICAL CORP) 25 March 1999 (1999-03-25)</p> <p>examples 4,5,10</p> <p>L: Priority</p> <p>-----</p>	1-72

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-72 in part

Present claims 1-72 relate to a method using an extremely large number of possible compositions. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compositions claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the methods using a composition described in the examples, with special emphasis on examples 60-66.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No
PCT/US 00/02620

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9640285	A 19-12-1996	AU 709562 B		02-09-1999
		AU 6270396 A		30-12-1996
		CA 2218541 A		19-12-1996
		EP 0831932 A		01-04-1998
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WO 9913919	A 25-03-1999	AU 9383098 A		05-04-1999
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